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Evidence for a Humoral Factor (or Factors) Concerned in Recovery from Radiation Injury: *A Review**

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The death of animals exposed to single total-body doses of ionizing radiations in the lethal range is assumed to be due to the failure of functional reconstitution of one or more of the tissues in the body (e.g., the hematopoietic system), and until recently the possibility of a specific approach to the management of the severe injury produced by such an exposure seemed far-fetched.

Prophylactic measures, such as pretreatment with estrogen (53) or cysteine (43), have been used to reduce radiation morbidity and mortality in experimental animals, but, while of great fundamental importance, they are not effective in enhancing recovery when given after the radiation exposure has been sustained. Experiments which may be termed "therapeutic approaches" to the problem have yielded results that have changed this rather discouraging picture to one of restrained optimism; these studies—some of which have been reported previously and others which are herein reported in the open literature for the first time—are briefly reviewed in the paragraphs to follow.

THE EFFECTS OF LEAD-SHIELDING THE EXTERIORIZED SPLEEN COMPARED TO SHIELDING OTHER PARTS OF THE BODY

Lead-shielding of the surgically exteriorized spleen (average weight, 0.1 gm.) of adult mice during exposure to 1,025 r total-body x-radiation markedly enhances survival (77.0 per cent[1]), compared to the same exposure without spleen-shielding (1.1 per cent[1]); none survive exposure to 1,100 r; but 55 per cent survive this dose if the spleen is shielded (23, 32). After exposure to 1,025 r, no anemia and only a transient leukopenia and thrombocytopenia appear in spleen-shielded mice, whereas pancytopenia and death follow exposure without spleen-shielding. Recovery of hematopoietic tissue in spleen-shielded mice occurs by 8 days, but no hematopoietic recovery is noted during this interval in unshielded mice (21). Recovery of the lymphatic tissue in the wall of the gastrointestinal tract in spleen-shielded mice parallels recovery of the hematopoietic tissue elsewhere. These observations prompted the author to postulate that the mechanism of recovery from radiation injury under these conditions was on a humoral basis and that the factor (or factors) responsible was produced by the cells of the protected tissue (17, 29, 33).

The survival of mice exposed to 1,025 r total-body x-radiation is approximately 30 per cent if part of the exteriorized liver (0.8 gm.), the exteriorized intestine (2.5 gm.), the entire head (3.0 gm.), or one entire hind leg up to the thigh (1.5 gm.) is lead-shielded during exposure. Without shielding, only 0.8 per cent survive this dose; with spleen-shielding, at least 76 per cent survive. Shielding one exteriorized kidney (average weight, 0.19 gm.) does not enhance survival. Recovery of the hematopoietic tissue, as judged by histopathologic study, is under way by 8 days in liver- or intestine-shielded animals, whereas after lead-shielding of the head, recovery of these tissues is delayed even longer, and the recovery of hematopoietic tissue is nil with kidney-shielding (21, 29, 30, 32).

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[1] These percentage figures vary throughout manuscript, because survival varies from one experiment to another.

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The amount of tissue shielded in the intestine and liver experiments is greater, respectively, by factors of 25 and 8 than in the spleen-shielding experiments. These findings cannot be interpreted with any certainty but do suggest that the potential production of a factor (or factors) by the intestine and the liver is not so great as that by splenic tissue but yet is sufficient to institute recovery early enough in a sufficient number of cells of the body to have a definite effect on survival.

It is conceivable that nearly all tissues of the body are capable of producing the factor (or factors) concerned in recovery from radiation injury, but certain tissues and, in particular, the hematopoietic system have a greater potential production per unit volume.

There are several differences between head- or limb-shielding and spleen implantation (to be described later) and spleen-shielding. According to generally accepted concepts, the reduction in volume dose when the head, limb, or intestine is shielded must be considered as playing a role in the reduction of mortality, since these structures represent a fairly large proportion of the body weight (15 per cent, 7.5 per cent, and 12.5 per cent, respectively). On the other hand, the shielded spleen weighs only 0.1 gm. (0.005 per cent body weight), and spleen implants weigh 0.010 gm. (0.0005 per cent body weight), which eliminates the volume-dose factor of shielded spleen or implanted spleen from consideration. The fact that the head, hind limbs, and intestine contain reticulo-endothelial tissue and other tissue of mesenchymal origin may be as important or more important than the volume-dose consideration.

The work of other investigators who have studied the effect of shielding various parts of the body on radiation mortality (1, 2, 6, 7, 10, 12, 49) will be discussed in the paragraphs on species differences.

Effect of Clamping Off Splenic Circulation during the Irradiation-Shielding Procedure

The survival of mice in which the circulation to the shielded spleen is clamped off during exposure of the animal to 1,025 r and in which the clamp is released immediately after irradiation is approximately the same as survival in animals with spleen-shielding without clamping (30–32). Histologic recovery of the hematopoietic system is the same as in the spleen-shielded animals without clamping on the splenic pedicle. This observation was convincing evidence that the presence of shielded tissue in the circulation was not required during the period of irradiation in order for survival to be enhanced and hematopoietic regeneration to proceed. Re-introduction of the spleen into the circulation after irradiation could thus be considered an effective postirradiation "therapeutic approach" to the problem.

None of eight mice survived which had the circulation to the spleen clamped off without simultaneous spleen-shielding during exposure to 1,025 r (16). In this experiment, however, the clamp was applied immediately before irradiation of the animals and released immediately after the completion of irradiation. In view of the observations of Jolly (35) and Dowdy (9), one might expect temporary clamping of the splenic pedicle during exposure of the mouse to 1,025 r to enhance survival if indeed the radiation injury sustained by the splenic tissue is sufficiently reduced by this procedure.

Further data, including histologic study of the spleens which have been clamped off during irradiation, are necessary before the reason for the ineffectiveness of this procedure can be resolved.

Effect of Splenectomy after Spleen-Shielding

Surgical extirpation of the initially shielded spleen at intervals after exposure of mice to 1,025 r total-body x-radiation shows that a beneficial effect (survival of greater than 70 per cent and early regeneration of hematopoietic tissue) has already been exerted if the shielded spleen is left intact in the circulation for 1 hour (32). Leaving the spleen in the circulation for longer periods, such as 6 hours, 24 hours, or 2 or more days, does not increase the percentage of animals surviving. In a previous communication (30) it was reported that, if splenectomy was performed within 10 minutes after the irradiation–spleen-shielding procedure, none survived. Further work, however, has shown that leaving the spleen in the circulation for as little as 5 minutes is sufficient to increase significantly the survival of mice exposed to 1,025 r (24). Full recovery of the blood-forming tissues is delayed longer in mice splenectomized 5 minutes after irradiation than in mice splenectomized 24 hours after irradiation. If the originally shielded spleen (whether or not the pedicle is clamped during the irradiation) is not removed, complete regeneration of hematopoietic tissue occurs earlier than in mice with splenectomy 24 hours after the shielding procedure (32). These facts indicate that the intact spleen may release enough of the factor in a few moments significantly to enhance survival but that, if left in the circulation longer, a greater and more rapid regeneration of hematopoietic tissue occurs.

OBSERVATIONS ON RELATIONSHIP OF AGE OF MICE TO EFFECT OF SPLEEN-SHIELDING ON SURVIVAL

Lorenz and associates (40) reported that 4-week-old strain A mice died within the first 8 days after exposure to 800 r x-radiation with spleen-shielding. The survival of adult mice (10 weeks of age or more) of the same strain, exposed to 900 r with spleen-shielding, was 95 per cent. Simmons and associates (48) investigated this problem in Carworth (CF #1) female mice exposed to 1,025 r with spleen-shielding and found that, whereas the survival of adult mice (10–12 weeks) was 76 per cent, the survival of younger mice was as follows:

No. of animals	Age (weeks)	With shielding (per cent survival)	Without shielding (per cent survival)
34	4– 5	38	
18	4– 5		0
138	5– 6	9.4	
18	5– 6		0
41	6– 7	63	
14	8– 9	64	
176	10–12	76	
122	10–12		0.8

These observations of Lorenz (40) and Simmons (48) suggest (*a*) that physiological aspects characteristic of the age influence the effectiveness of the shielded spleen to enhance survival or (*b*) that the shielded spleen produces less of the factor under discussion before maturity. The former suggestion seems the more likely, since the transplantation of spleens from immature mice of these same age levels to adult mice exposed to 1,025 r total-body x-radiation is about equally effective in enhancing survival (30–32).

EFFECT OF TOTAL-BODY EXPOSURE TO 1,025 R WITH 200 R TO THE SPLEEN ON SURVIVAL AND ON REGENERATION OF THE HEMATOPOIETIC SYSTEM

Mice have been exposed to 1,025 r total-body x-radiation and the spleen has been given various increments of the total-body dose. Doses up to and including 200 r may be given to the spleen at the same time as 1,025 r total-body exposure without reducing survival below the 75 per cent, which is expected from the earlier spleen-shielding studies (32, 34). In contrast to animals with spleen-shielding, and thus no irradiation of the spleen, these animals become moderately anemic and develop a severe leukopenia that persists beyond the twelfth day. Histological studies show that recovery of the blood-forming tissue is qualitatively complete by 10–12 days (32). Even with dosages of 400 r, 500 r, or 600 r to the spleen and 1,025 r to the body, survival is significantly higher (59, 50, and 34 per cent, respectively) than in mice exposed to this dose without spleen-shielding (1.1 per cent). These data indicate that the capacity of the splenic tissue to elaborate the factor is still partially retained or recovery of the tissue in the spleen that produces the factor occurs early enough to enhance survival, even with doses as high as 600 r to the spleen and 1,025 r to the body. These observations tend to add support to the hypothesis that the factor (or factors) responsible for recovery from radiation injury under these circumstances is derived from more primitive but more "radioresistant" cells, such as reticular cells, rather than from "free" cells such as lymphocytes, granulocytes, and the like. The spleen is, for all practical purposes, devoid of these "free cells" after a dose of 500 r, whereas the basic reticular network remains "histologically" intact. It is interesting that with a lethal dose to the body (1,025 r) and an LD_{50} (500–600 r) to the spleen *circa* 50 per cent of the animals survive.

EFFECT OF POSTIRRADIATION SPLEEN TRANSPLANTATION ON RECOVERY FROM IRRADIATION

Transplantation of spleens (total weight, 10–100 mg.) from baby or adult mice into the peritoneal cavity of mice within 2 hours after exposure of the recipient adult mice to 1,025 r total-body x-radiation significantly increases the survival (*circa* 50 per cent) of the irradiated mice and hastens regeneration of hematopoietic tissue (30–32). The donor spleens are simply dropped free into the peritoneal cavity. *Transplantation of spleens into the peritoneal cavity of mice 1 or 2 days after exposure to 1,025 r total-body x-radiation likewise enhances survival* (circa *25 per cent*) *but not as effectively as earlier transplantation* (30, 34). Implantation of muscle into the peritoneal cavity after exposure of mice to 1,025 r total-body x-radiation has no beneficial effect on survival.

If splenectomy is performed in mice prior to irradiation, followed by the implantation of fresh spleens into the peritoneal cavity after irradiation, survival is enhanced, indicating that the animal's own spleen is not required to make the transplant effective (18). Surgical removal of the transplanted spleens from the peritoneal cavity of mice 1 and 2 days after the irradiation-transplant procedure has invariably been followed by death of the animals. Gross and microscopic observations on mice surviving the irradiation-transplant procedure reveal that the implanted spleen or spleens have vascularized and eventually appear as normal splenic tissue. Revascularization and reconstitution of the implanted spleen is usually well under way by the sixth day after implantation. Transplantation of splenic tissue 2

days after irradiation is admittedly less effective in enhancing survival of mice exposed to 1,025 r than earlier transplantation, but it has not been determined when a state of irreversibility has been reached. Actually, if establishment of a vascular supply to the transplant is essential to the manufacture and transport of the factor (or factors) in question, one might venture a conservative guess that supplying an optimum amount of the factor to mice as late as 6 days after exposure to 1,025 r would still significantly increase the survival.

Effect of Suspension of Mashed Embryos on Recovery from Radiation Injury

Intraperitoneal administration of a suspension of 12-day-old mouse embryos, prepared by forcing the embryos through an 18-gauge stainless steel mesh, and of such a consistency as to permit delivery through an 18-gauge hypodermic needle, is effective in enhancing survival of mice exposed to 1,025 r total-body x-radiation (19). This suspension, prepared in the cold with or without the addition of normal physiological saline or buffered saline, when given intraperitoneally in a dosage of from 0.5 to 1.0 ml. 2–6 hours after irradiation of the recipient resulted in 30 per cent survival in 95 animals. Suspensions of baby or adult spleens, prepared and administered in a similar manner, have thus far been ineffective in enhancing survival of mice exposed to 1,025 r but have been effective in enhancing the survival of mice exposed to 800 r (20). Chick embryo suspensions (age of embryos, 11–14 days), prepared and administered in a similar manner to the mouse embryo suspensions, have been reported by Marks and Brues[2] to be ineffective in enhancing the survival of mice exposed to dosages of x-radiation in the LD_{50} range or above.

The factor (or factors) in the embryo or spleen suspensions responsible for recovery from radiation is probably the same as that which is responsible for the effectiveness of the spleen-shielding and spleen implants. Two possible explanations for the effectiveness of cell suspensions are obvious: (*a*) that the cells in the suspension quickly implant and begin elaboration of the factor or factors effective in initiating tissue regeneration throughout the body or (*b*) that the peritoneal cavity serves as an incubator which allows the cell suspension to remain alive and to elaborate the factor or factors responsible for increased survival and tissue regeneration in the irradiated mice.

[2] Unpublished data of E. K. Marks and A. M. Brues.

Effect of Postirradiation Parabiosis on Survival from Radiation Injury

Brecher *et al.* (8) have recently reported that approximately 50 per cent of rats exposed to 700 r total-body x-radiation survive if joined to normal nonirradiated litter-mates within a few hours after irradiation. None of the controls given the same dose of irradiation survived the 28-day period of observation. Hematopoietic regeneration was more rapid in the irradiated rats with a parabiont than in the irradiated controls. These findings, like those of Lorenz (42) reported below, corroborate Jacobson's previously reported observation that effective postirradiation "therapy" is a reality. Like the embryo suspension experiments described above, however, the experiments of Brecher do not point out the cellular source or the identity of the effective factor (or factors) concerned.

Relationship of the Quantity of Shielded or Implanted Splenic Tissue and Survival of Irradiated Mice

Two separate observations indicate that a definite relationship exists between the quantity of implanted or shielded tissue and the effect as measured by survival from irradiation (32).

1. The transplantation of two spleens (wt., *circa* 5 mg.) from 7- to 12-day baby mice into the peritoneal cavity of mice immediately after irradiation (1,025 r) does not enhance survival, although the transplantation of four spleens (wt., *circa* 10 mg.) is effective in reversing the process in time to allow recovery of the animal (32).

In the regular spleen-shielding technic the main splenic pedicle is left intact, but a small blood vessel at the distal tip of the spleen is severed to facilitate exteriorization and lead-shielding of the spleen. Invariably, from one-fourth to one-half of the spleen proximal to the severed vessel becomes infarcted and undergoes liquefaction necrosis. If this vessel is not cut so that the whole spleen is shielded and remains intact, 100 per cent of the animals survive exposure to 1,025 r rather than the expected 77 per cent. In fact, with a total-body exposure to 1,300 r, only 3.4 per cent of animals survive if the distal vessel of the spleen is cut during the shielding procedure, whereas 26.9 per cent survive this exposure with spleen-shielding if the distal vessel is not cut (32). This observation, like the one described above, indicates that the quantity of the "factor" being produced is directly related to the number of cells available in the shielded or implanted tissue, and the amount of the "factor" available determines the survival of the animal. It would appear that the repair

process must be initiated in a minimum number of cells in the body of the irradiated animal to insure survival of the animal exposed to dosages of 1,000 r or more.

Evidence from Histologic Studies Supporting Humoral Theory of Cell Regeneration

Rabbits exposed to 800 r or 1,000 r with spleen-shielding or appendix-shielding show histologic evidence of beginning recovery of hematopoietic tissues on the fourth postirradiation day, whereas in animals thus exposed without shielding evidence of regeneration is delayed for 8 or more days (26, 28). Mice exposed to 1,025 r with spleen-shielding likewise show histologic evidence of recovery of the hematopoietic tissue on the fourth postirradiation day; in fact, recovery is essentially complete by 8 days. Mice irradiated without shielding have no evidence of recovery or show "spotty foci" of beginning regeneration in the bone marrow on about the tenth to twelfth day. Death of all mice without spleen-shielding occurs by the fourteenth day. In none of the animals examined, including those on the fourteenth day, has hematopoietic regeneration been other than focal. In these mice and rabbits which had spleen-shielding, regeneration may occur from the scattered "free cells" in the lymphatic tissues and bone marrow that survive the radiation, *but heteroplastic regeneration from reticular cells is prominent.* Thus, colonization from the shielded tissue, followed by repopulation by multiplication of these colonized cells, if a factor at all, is only one aspect of the recovery process. The shielded tissue in some way restores the functional capacity of the reticular cells to repopulate the hematopoietic tissues. The shielded tissue may likewise restore the functional capacity of the residual "free cells," which are not destroyed by irradiation, to multiply and thus repopulate the hematopoietic tissues. Cells coming from the shielded or implanted tissue cannot at the moment be distinguished from these residual free cells. If the cells which migrate out from the shielded tissue do "lodge" in hematopoietic tissue, then it is possible that they also contribute by division and multiplication and also by elaboration of the factor (or factors) under discussion in this dissertation. In a previous report (26, 28) it was shown that recovery of hematopoietic tissue was more rapid in rabbits which had shielding of the exteriorized appendix during exposure to 800 or 1,000 r total-body x-radiation than in animals similarly exposed without appendix-shielding. The shielded appendix does not become the site of ectopic formation of such cells as erythroblasts, megakaryocytes, or granulocytes. If recovery of the hematopoietic system could be attributed primarily to migration of cells from the shielded tissue and subsequent multiplication of these cells, then the general concept of the unitarian school of hematology would have additional support, since the appendix is exclusively a lymphatic organ.

Species Differences in Effect of Spleen-Shielding on Recovery from Radiation Injury

A significant percentage of mice (31 per cent) with spleen-shielding survives a dose of 1,300 r total-body x-radiation, which is more than 500 r above the 30-day LD_{99}. Recovery of the blood-forming tissue is rapid in these mice and by hematologic examination is shown to be well under way by 8 days (25). The effect of spleen-shielding on the survival of rats is also definite but less spectacular than in mice (34). The effect of spleen- or appendix-shielding on the survival of irradiated rabbits has not been carefully studied, but it is clear that no such enhancement of survival occurs as is observed in spleen-shielded mice or rats. Spleen- or appendix-shielding in this species (rabbit) during exposure to 800 r or 1,000 r appears not to affect survival appreciably, even though regeneration of blood-forming tissue precedes the recovery of this tissue in the animals without spleen- or appendix-shielding (32). These species differences cannot be adequately evaluated at the present time, since, for example, considerable differences may exist between rabbit and mouse spleen in terms of the potential production of the factor (or factors) involved in survival or early regeneration of hematopoietic tissue.

Allen (2) has reported that 450 r total-body x-radiation is invariably lethal to dogs. With head-shielding, however, the mortality of this dose is reduced to 75 per cent, and other aspects of the usual postirradiation syndrome, such as hemorrhage and evidences of infection, are greatly reduced or absent. Further work on the dog, comparing the relative effectiveness of shielding such parts as the head, spleen, intestine, limbs, and liver, will be of interest if for no other reason than to obtain base-lines on the potential effectiveness of these tissues for comparison with mice, rats, and rabbits and to accumulate some facts on the potential production of the factor on a tissue weight basis, type of tissue shielded, etc. In this connection, the work of Bond *et al.* (7) in which the abdomen of rats was shielded with lead during total-body x-radiation is of interest. The LD_{50} for these abdominally-shielded rats was 1,950 r, compared to *circa* 700 r for nonshielded control rats. For very practical reasons it would be of interest more

precisely to determine the relative importance of the various abdominal tissues in enhancing survival on a weight basis and more adequately to assess the volume-dose factor. This has been done to some extent by Gershon-Cohen and associates in rats (12) and by Jacobson *et al.* (21, 29, 30, 32) in mice, but further data in all species are needed. As was pointed out by Bond (7), the radiosensitivity of the part of the body irradiated may be more important than the gram-roentgens sustained by the balance of the body. To this must be added the fact that the actual or potential production of the factor (under consideration in this paper) by the shielded or nonirradiated tissue may be more important in determining survival of the animal than the radiosensitivity of the tissue in the radiation field and, within certain limits, more important than the gram-roentgens sustained by the balance of the body.

Effect of Postirradiation Injection of Homologous Bone Marrow on Survival of Irradiated Mice

Lorenz *et al.* (37, 41, 42) have recently shown that, whereas 900 r is the LD_{99} for genetically homogeneous hybrid LAF_1 mice, approximately 75 per cent survive this dose if bone marrow from normal nonirradiated mice of the same strain is injected intravenously within an hour after the irradiation. If the bone marrow is administered intraperitoneally, survival is slightly less (*circa* 50 per cent). The author estimates that the total weight of the injected marrow is approximately 1.5 mg. The recovery of the hematopoietic tissue of the bone marrow-treated mice as in the spleen-shielded, spleen-implanted, or embryo suspension-injected mice is hastened. Jacobson *et al.* (16) have corroborated Lorenz's finding. The survival of Carworth (CF #1) female mice exposed to 900 r and given homologous bone marrow intravenously immediately after irradiation was approximately 50 per cent. Rekers (44, 45) and Talbot (51, 52) have reported negative or equivocal beneficial effects on the radiation syndrome in dogs and rats, respectively, after bone marrow administration. In a preliminary report by Hilfinger *et al.* (14), normal rabbit bone marrow was emulsified in serum and injected intravenously into rabbits after exposure to dosages from 1,000 r to 1,400 r total-body x-radiation. A more transient leukopenia was observed in the bone marrow-treated rabbits than in the control irradiated rabbits.

The suspension of bone marrow which Lorenz has injected contained mature and immature free cells, such as granulocytes and megakaryocytes, as well as free and fixed macrophages, reticular cells, and endothelial tissue. It seems likely that the cells injected establish themselves as scattered foci of hematopoietic tissue and produce a factor (or factors) responsible for survival of the animal which is identical with that postulated in the spleen-shielding, spleen-implantation, and embryo-suspension experiments. No data are as yet available to compare adequately the relative effectiveness of splenic tissue and bone marrow in enhancing survival from radiation injury.

Effect of Heterologous Transplants and Cell Suspensions on Recovery from Radiation Injury

Jacobson and associates (22) have reported a preliminary experiment in which rabbits were exposed to 800 r total-body x-radiation and, following exposure, from four to thirteen spleens, freshly obtained from young mice, were inserted into the peritoneal cavity of the rabbits. No attempt was made to study the effect of this procedure on the survival of the irradiated rabbits. At intervals after the irradiation-spleen-transplant procedure the rabbits were sacrificed, and the hematopoietic tissues as well as the donor spleens were removed for histologic study. The postirradiation intervals studied were 6, 8, 10, and 12 days. At the time of sacrifice it was obvious on gross examination that the donor spleens were firmly attached to the spleen of the rabbit or the surrounding omentum, and many or all of the donor spleens were viable. Microscopic study of the donor spleens revealed even at the 12-day interval that vascularization had occurred, viable cells including lymphocytes and megakaryocytes were in abundance, and in some of the donor spleens the basic splenic architecture was still clearly evident. On comparison of the extent of regeneration of the hematopoietic tissues of the irradiated rabbits which had intraperitoneal mouse spleen transplants with the irradiated controls, it was found that only on the twelfth postirradiation day was a difference demonstrated. At this interval regeneration of hematopoietic tissues was normal or hyperplastic in two of four rabbits which had spleen transplants, whereas only relatively little evidence of regeneration was found in four control irradiated rabbits. This finding may be a coincidence or a reaction to the foreign transplant and should therefore not be considered as evidence indicating the effectiveness of heterologous tissue to hasten recovery from radiation injury.

Lorenz has reported evidence of the effectiveness of heterologous tissue transplants on recovery from radiation injury (38). Within an hour after exposure of mice to 900 r (LD_{99}) of total-body x-

radiation, approximately 25 mg. of freshly aspirated guinea pig bone marrow (in buffered saline) was injected intravenously into the irradiated mice. None of the control irradiated mice survived, but 40 per cent of the irradiated mice which received intravenous guinea pig bone marrow suspension survived the 28-day period of observation. The number of animals used by Lorenz was small, and the results must be considered preliminary. The survival of this strain of mice injected with homologous bone marrow (1.5 mg.) after exposure to 900 r is *circa* 75 per cent.

The report of Lorenz (38), if it can be corroborated, probably should be considered as conclusive evidence that the factor (or factors) supplied by spleen or bone marrow, which so significantly enhances survival, is indeed a humoral substance (or substances).

It is not likely that heterologous spleen transplants or heterologous bone marrow injection, if indeed they are effective at all, produces its effect by seeding the mouse or rabbit hematopoietic tissue with cells which by multiplication repopulate the bone marrow. It seems more likely that this heterologous tissue lives, at least temporarily, in its new environment and produces a substance (or substances) that aids in recovery from the radiation injury.

Effect of Combined Prophylactic and Therapeutic Measures on Survival from Radiation Injury

The fact that a reduction in the mortality of animals exposed to lethal dosages of total-body x-radiation could be effected by pretreatment with estrogens (53) as well as by spleen-shielding (21) suggested to Simmons (47) that these two measures might produce an additive effect on survival. Accordingly, he tested this hypothesis and found that (*a*) mortality of mice exposed to 1,025 r total-body x-radiation was 100 per cent, (*b*) 61.5 per cent of mice survived this dose if estrogens were given prior to irradiation, (*c*) 82.3 per cent survived if the spleen was shielded during exposure to 1,025 r, and (*d*) 100 per cent survived 1,025 r if the technics of pretreatment with estrogens as well as spleen-shielding were employed. Bethard (4), employing the same general approach, found that cysteine and spleen-shielding similarly had an additive effect on survival of mice exposed to x-radiation. Furthermore, it was found that, when the technics of pretreatment with estrogens and cysteine and spleen-shielding during irradiation were all combined, an additive effect of all three on survival was observed (5). Jacobson found that pretreatment with cysteine, followed by 1,025 r total-body x-radiation, and postirradiation intraperitoneal transplantation of normal spleen were also additive in enhancing survival (16). Cysteine fails to produce any enhancing effect on survival of irradiated mice when given after 1,100 r total-body x-radiation, whether or not the mice had spleen-shielding during irradiation. These studies, while very interesting, shed no light on the obvious question of whether or not the pretreatment or prophylactic technics are related to the "therapeutic" technics from the standpoint of mechanism.

Studies on Antibody Formation

The fact that a single total-body exposure to x-rays inhibits antibody formation is well documented (13). It was recently shown that, if the spleen or the appendix of the rabbit is surgically exteriorized and shielded with lead during total-body exposure to 800 r, the capacity to form antibodies to a particulate antigen injected 24 hours after irradiation is retained (28). In another series of experiments this observation was carried a step further (25, 27). Rabbits were exposed to dosages of 800 r or 500 r total-body x-radiation with spleen-shielding. Twenty-four hours later the spleen was removed surgically. After another 24-hour period (48 hours after irradiation) a particulate antigen (sheep red cells) was given intravenously. The capacity of these animals to form antibodies (anti-sheep cell hemolysin) was compared to that of various control groups given the same antigen at the same time relative to the irradiation as the experimental animals described above. The capacity to form antibodies to the injected antigen was retained in the rabbits given 800 r or 500 r total-body x-radiation which had spleen-shielding during irradiation, the spleen left intact in the circulation for 24 hours and then removed surgically even though the antigen was given 24 hours after splenectomy and 48 hours after irradiation. The facts that these rabbits retained the capacity to form antibodies, even though hematopoietic tissues in the body were as yet atrophic, and that control rabbits exposed to the same dose did not retain this capacity are considered to be results of the functional restoration of cells in the body (such as free and fixed macrophages and reticular cells) by a humoral (noncellular) substance entering the general circulation from the originally shielded spleen during the 24 hours prior to splenectomy.

GENERAL DISCUSSION

In view of these observations, it seems extremely unlikely that cell migration from the shielded or transplanted tissue and subsequent

proliferation of these cells account for the reconstitution of hematopoietic tissues and increased survival of irradiated animals or that neutralization of some "toxin" produced by irradiation can account for these findings. Perhaps neither of these possibilities can be positively excluded on the basis of the evidence presented, but the evidence strongly suggests that the factor (or factors) responsible for recovery from radiation under these circumstances is noncellular and *may be required only for the initiation of the repair process. The factor (or factors) may be quite labile or, as is more likely, may be produced in an effective quantity only by living cells.* These cells may be present in shielded or implanted tissue or may have migrated out, but wherever they are they are probably producing the factor under discussion. *The factor may be a single substance such as an enzyme or coenzyme necessary for the functional reconstitution of many different cell types in the several organ systems or several different factors may be concerned.* Salisbury (46) found in a small number of dogs that early direct cross-transfusion between irradiated (LD_{90} x-ray) and nonirradiated dogs significantly reduced mortality, reduced the severity of the expected hematopoietic depression, and reduced the severity of the usual clinical signs of irradiation sickness. Salisbury's experiments (46) should be expanded in numbers of animals and more adequately controlled to make the significance of the experiment more clear-cut.

The fact that 75 per cent of mice that have lead-shielding of the spleen during exposure to 1,025 r and then splenectomy 1 hour after the irradiation–spleen-shielding procedure survive would lead one to expect early administration of whole blood to be effective as well. This seems logical since whatever the spleen accomplishes under these circumstances must have been via the blood stream. That the factor (or factors) must be supplied early is also strongly supported by the fact that in mice given a lethal dose of radiation spleen transplants are more effective on the day of irradiation than on the second day after irradiation. In other words, supplying "the factor" responsible for initiating the functional repair in some unknown minimum number of cells throughout the body must be accomplished early enough to reverse the processes that ordinarily end in death of the animal. Once the factor is adequately supplied, as is clearly demonstrated by the splenectomy experiments, the process of repair is initiated, yet histologic evidence of repair or regeneration is not apparent for 4 or more days.

Indirect transfusion initiated on the fourth postirradiation day has been reported to be unsuccessful in significantly increasing survival of x-radiated dogs (3). This apparent failure of whole blood transfusions to enhance survival effectively and reduce morbidity is understandable if one assumes that (*a*) the amount of the "factor" (or factors) present in the blood per unit volume is small, and therefore relatively large amounts of blood would be necessary to initiate effectively the recovery process, or (*b*) the factor (or factors) was administered too late after exposure of the recipient to initiate the repair process in time and widely enough in cells of the body to have a critical effect on morbidity and survival. The preliminary experiments of Swisher *et al.* (50) indicate that the morbidity of dogs exposed to dosages of x-radiation in the mid-lethal range is reduced if as little as 250 cc. of whole blood collected in ACD solution from a compatible donor is administered to irradiated dogs shortly after exposure. The lability of the factor is as yet unknown. The ineffectiveness of cell-free extracts obtained from extirpated splenic tissue or embryos may only indicate that too small an amount of the factor is present or too small an amount of the factor is obtained in the extracts from these tissues by present methods. A method of preservation or concentration of the factor (or factors) or a more sensitive method of assay may be necessary before a positive result can be obtained by cell-free extracts. If the factor is present in whole blood in a concentration sufficient to alter the radiation syndrome even when given in relatively small amounts soon after irradiation of the recipient, then varying the conditions and methods of the administration of whole blood may supply important clues to the identification of the factor (or factors).

Any attempt to relate these findings to problems other than radiation injury would be premature at this time, but speculation is intriguing. It is a well known fact that in cases of myelogenous leukemia in the human being, x-radiation of the spleen is often sufficient to produce a hematologic and clinical remission. One might therefore suggest that these findings in animals, as reported above, are at variance with those observed in human leukemia. However, the spleen in myelogenous leukemia cannot be considered normal, and further study will be required before this apparent paradox can be resolved. It has been reported by Furth (11) and by Lorenz *et al.* (39) that the incidence of ovarian tumors was markedly increased in mice that had been given total-body radiation. Lick (36) reported, however, that if one ovary is lead-shielded and the balance of the body including the other ovary is irradiated, the incidence of ovarian tumors is significantly reduced. He postu-

lated that some factor from the nonirradiated ovary prevents tumor formation in both the irradiated and nonirradiated ovary. Lick's findings are undoubtedly on a hormonal basis and therefore may be quite different from the findings discussed concerning mice with spleen-shielding or implants. A recent report by Hollcroft and Lorenz (15) has perhaps more direct bearing on the problem related in this paper. These authors were studying the effect of irradiation on a transplanted lymphosarcoma in mice. The tumor grew slowly locally and killed the animal in 30–40 days. A dose of 400 r total-body x-radiation, including the tumor, failed to kill the tumor; local irradiation of the tumor alone with dosages up to a total of 1,300 r likewise was ineffective. A single dose of 800 r to the whole body (including the tumor) but with the spleen shielded effected a cure of the tumor in a high percentage of the animals so treated. These authors suggested that the "toxicity" produced by total-body irradiation was responsible for the effective suppression of the tumor; spleen-shielding merely made it possible for the mice to survive a dose that would ordinarily kill 100 per cent of animals irradiated without spleen-shielding.

CONCLUSIONS

A brief review of data is presented which conclusively shows that survival of laboratory animals exposed to a single lethal dose of total-body x-radiation can be significantly increased by measures instituted after the radiation injury has been sustained. These measures involve spleen-shielding, intraperitoneal implantation of splenic tissue, and intraperitoneal implantation of embryo suspensions, intravenous or intraperitoneal injection of homologous bone marrow suspensions, and related technics. The evidence presented indicates that the factor (or factors) responsible for recovery from radiation injury under these conditions is likely a humoral (noncellular) substance (or substances) produced by the living cells of the shielded, implanted, or injected tissue that is capable of instituting recovery of certain tissues of the body vital to the survival of the animal. If further experiments indicate that heterologous transplants or injections of tissue suspensions are effective, the possibility of seeding or colonization by cells of the shielded or implanted tissue as a factor in the reconstitution of the depleted blood-forming tissues is reduced to one of secondary importance for the purposes of this discussion. It is admitted, however, that a possibility exists that the shielded or implanted tissue may produce its effect by some detoxification process.

The identity of the humoral factor (or factors) under discussion is as yet unknown. Nor is it known from which cells of the body the factor (or factors) originates. It is produced or made available in an effective quantity by minute amounts of hematopoietic tissue or so-called reticulo-endothelial tissue, whereas larger quantities of tissue such as kidney have thus far proved ineffective. With the assay methods now in use living cells appear to be necessary in the material administered to alter effectively the radiation syndrome and promote recovery. It is firmly established that the factor (or factors), when made available to irradiated animals, brings about an early recovery of the blood-forming tissue. This recovery may be directly as well as indirectly responsible for the enhanced survival of irradiated animals. It may be that the factor (or factors) is involved in the orderly regeneration or functional reconstitution of yet other cells, tissues, or organ systems of the body which are important, if not vital, to survival of the irradiated animal.

The implications of these findings for problems of radiation injury and radiation therapy are obvious, but their application to medicine in general and to neoplastic and non-neoplastic diseases of the blood-forming tissue in particular requires exploration.

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The Hyaluronidase Content of Necrotic Tumor and Testis Tissue*

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The occurrence of a substance in tumor tissue which causes either an increased spread of fluids injected intradermally or the depolymerization of hyaluronic acid *in vitro* has been discussed by several authors. Duran-Reynals and Stewart (5) found a "spreading factor" in eleven of 23 human carcinomas, but in thirteen sarcomas they found no such effect. Boyland and McClean (2) showed that the activity of the "diffusing factor" present in nearly all the rapidly growing mammalian tumors they investigated was approximately proportional to the rate of growth. Chain and Duthie (3) found no hyaluronidase (HYAS) activity in Jensen sarcoma. Gibertini (7) observed hyaluronidase activity in four of 23 human tumors. Pirie (16) demonstrated hyaluronidase activity in some of the animal tumors he investigated but not in Jensen sarcoma. McCutcheon and Coman (14) reported that extracts from nine of ten human carcinomas had "diffusing activity." Employing the viscometric method, they found that extracts from six of seven carcinomas showed hyaluronidase activity. Dux, Guérin, and Lacour (6) investigated seventeen human tumors of various types, two of which showed hyaluronidase activity. However, the possibility that bacterial contamination may be the source of the enzyme activity cannot be excluded. Among nineteen cases of transplantable spontaneous animal tumors of various types, they found hyaluronidase activity in only three. Recently, Kiriluk, Kremen, and Glick (10) reported the absence of hyaluronidase activity in human benign and malignant tumors and in mouse mammary adenocarcinoma. They suggested that the positive results of the previous authors were caused by bacterial hyaluronidase present in infected tumor tissue.

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MATERIAL AND METHODS

In the present study Walker rat carcinosarcoma 256 and Ehrlich mouse carcinoma, both subcutaneously implanted, were used. The transplantation of the tumors was carried out under aseptic conditions, as was the excision, which was performed 7–12 days later with the Walker tumors and 15 days later with the Ehrlich sarcoma.

Before the tissue extracts were prepared, the sterility of each of the tumors was tested. The culture media chosen were liver broth and 10 per cent blood-agar. Both aerobic and anaerobic tests were made. If a tumor showed any *trace* of bacterial contamination, it was not used for further experimentation. The same technic was used in testing the sterility of the testicular and tumor tissue incubated *in vitro* (vide infra). A test for bacterial contamination was also carried out at the end of the incubation of the tissue at 37°, and, if any bacterial growth was then found, the sample was rejected.

Samples of the capsular, the living, and the necrotic parts of the tissue were weighed, after careful separation. The quantity of the tissue employed varied between 0.1 and 1.0 gm., depending upon the size of the available homogeneous material. After thorough homogenization in an all-glass grinding tube, at +2° to +4° C. for 5–10 minutes, the homogenates were diluted with 0.15 M NaCl solution. The dilution factor for tumor tissue homogenates was 1:10 (1 gm. tissue: 9 ml. solution), and for testis tissue homogenates it was 1:300, 1:500, or 1:1500.

Tissue extracts can be frozen without altering the measurable hyaluronidase activity. Swyer (17) has shown that, when spermatozoa suspensions are frozen, the hyaluronidase activity rises. Similar findings were made with the tissues employed in this study. However, it was observed that, when frozen tissue was used, the reproducibility of the experimental results was markedly impaired. Therefore, precautions were taken to avoid any freezing of the tissue before homogenization.

After a short period of shaking (3–5 minutes), the tubes were centrifuged 15 minutes at a speed which gave a relative centrifugal force of $600 \times g$ at the tip of the glass tube, and the extracts were filtered through paper. This operation was not carried out under sterile conditions. Since the preparation and incubation periods were relatively short (time from death of animal to viscosity determination being a maximum of 2 hours), and, since low temperatures were also employed, the possible contamination by air-borne bacteria during these processes would not be expected to influence the results. Samples of substrate which were allowed to stand in air at 2°–4° C. under the same experimental conditions showed no change in viscosity. Furthermore, although no experiments were conducted on the tissue extract itself, it is extremely improbable that, under the conditions employed, any significant bacterial growth would occur. Actually, tissue extracts kept for 12 hours at +2° to +4° C. show no significant deviation in hyaluronidase activity from that of the normal experimental material. In addition, prolonged standing (5 days) of the tissue extract at this low temperature leads to a decrease in hyaluronidase activity rather than an increase, as would be expected in the presence of a hyaluronidase-producing bacterial population.

The substrate used was an extract of human umbilical cord purified by the chloroform-amyl alcohol method described in detail by Blix and Snellman (1). Hyaluronidase (HYAS) activity was measured viscometrically at 36° C. An Ostwald viscometer was used, with an outflow-time for water of 26 seconds at 36° C. The reaction mixture consisted of 4 ml. substrate +1 ml. tissue extract. The parallel control to determine the initial viscosity of the reaction mixture contained the same quantity of substrate +1 ml. heat-inactivated tissue extract (60° C. for 3 minutes). This control reaction mixture showed a constant viscosity at 36° C. for 4–5 hours. The solution was found to be sufficiently buffered by the extract, and measurement with a glass electrode showed it to have a pH value of 6.5 ± 0.1. This variation in pH has no significant influence on the HYAS activity. No difference in pH was found between the extracts of necrotic and living tissues.

To compare the HYAS activity of different extracts we used the "half viscosity time." This is the time required to diminish the viscosity of the hyaluronic acid to half its original level. Madinaveitia and Quibell (11) were the first to show that the concentration of testis HYAS is inversely proportional to the half viscosity time, this relationship holding only over the range of salt concentration between 0.05 M and 2.0 M NaCl. This was confirmed by McClean and Hale (12), Haas (8), and Tobin *et al.* (19). The observations of other authors (Hale [9], Swyer and Emmens [18], and Dorfman [4]) show that this relationship is dependent upon such factors as pH, type of buffer, ionic strength, substrate concentration, and source of the enzyme. Chart 1 indicates that, under the conditions of the present experiments, the relationship between the dilution of the tissue extract and the half viscosity time is linear.

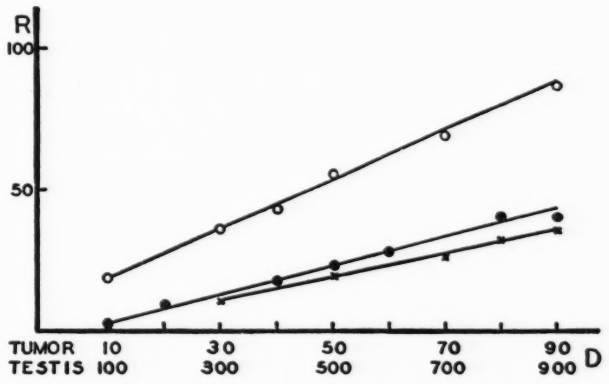

CHART 1.—Relationship between dilution of the tissue extract and the "half viscosity time."

R = "Half viscosity time" in minutes. Reaction time for half viscosity level of 4 ml. sodium hyaluronate in the presence of 1 ml. tissue extract.

D = Total volume of 0.15 M NaCl solution in ml. used for homogenization and dilution of 1 gm. tissue.

Open circle = Living part of tumor tissue (Walker carcinosarcoma 256).

Full circle = Necrotic part of tumor tissue (Walker carcinosarcoma 256).

Cross = Testicular tissue of the rat.

TABLE 1

COMPARISON OF HYALURONIDASE ACTIVITY IN LIVING AND NECROTIC PARTS OF RAT CARCINOSARCOMA*

Case no.	Half viscosity time (min.) Living tissue *a*	Necrotic tissue *b*	*a/b*
1	17.0	2.8	6.0
2	17.0	3.2	5.4
3	18.0	3.1	5.8
4	21.0	3.5	6.0
5	31.0	5.0	6.2
6	40.0	7.5	5.3
7	50.0	10.0	5.0
8	90.0	12.5	7.2
9	90.0	13.7	6.6

* 1 Gm. tissue homogenized in 9 ml. of 0.15 M NaCl.

RESULTS

Walker carcinosarcoma.—HYAS was found in all the tumors investigated, but its activity varied in the individual samples (Table 1). It is apparent that the enzymatic activity of the necrotic part of

the tumor is in all cases much greater than that of the living part. The HYAS activity completely disappeared after 3 minutes of heating at 60° C. To determine whether some low molecular weight substance (e.g., ascorbic acid, glutathione, cysteine, etc.) is responsible for the depolymerizing property of the tumor or testis extracts, they were dialyzed against 0.15 M NaCl at +4° C. for 48 hours. One part of each extract was stored under the same conditions without dialysis. The hyaluronic acid depolymerizing activity was still present after dialysis. There was no significant difference between the "half viscosity time" of the dialyzed and the control extracts. These results support the evidence that the depolymerization effect is not due to low molecular substances.

TABLE 2

HYALURONIDASE ACTIVITY OF NORMAL AND NECROTIC TESTICULAR TISSUE

Half viscosity time (min.)		Time after ligation	
Control testis	Necrotic testis	(days)	Dilution
17.5	0.6	2	1:500
60.0	17.5	2	1:1500
12.0	1.5	3	1:300
11.6	1.2	5	1:300
10.0	0.6	5	1:300
8.2	0.8	5	1:300
19.0	7.6	9	1:500

TABLE 3

HYALURONIDASE ACTIVITY OF TUMOR TISSUE DURING ASEPTIC AUTOLYSIS *in Vitro**

Time of autolysis (days)	Half viscosity time (min.) No. 1	No. 2
0	85.0	60.0
1		8.2
2	53.0	
6	10.2	3.8
7	4.5	2.9

* 1 Gm. tissue homogenized in 9 ml. of 0.15 M NaCl.

The Ehrlich carcinoma.—These tumors did not show any HYAS activity in either the living or the necrotic parts.

Necrosis of the testis.—To determine whether a higher enzyme content of the necrotic tissue would occur in other tissues containing hyaluronidase, a sterile necrosis of the testis was investigated. All the blood vessels of one of the testes of adult white rats (weighing 200–250 gm.) were aseptically ligated. At different times after the operation, comparison was made of the HYAS activity in the ligated and the unligated testes. Histologic controls showed the fully normal structure of the latter and the degree of necrosis in the former. Sterility controls were carried out as described above. The method of determining the HYAS activity was the same as that previously described. The results are presented in Table 2. The necrotic testes had, in all cases, higher HYAS activity than did the normal ones. The water content was diminished by only 4 per cent during 5 days' necrosis, so the HYAS activities of the two types of tissues calculated per unit wet weight are comparable.

Necrosis in vitro.—The next question was whether this increase in HYAS activity would also occur in necrosis of tumor and testicular tissue *in vitro*. Testes and the living part of tumors were aseptically removed, put into sterile glass vessels, well closed so that no moisture should be lost, and incubated at 37° C. for different periods of time. Tests (see methods) to exclude the presence of bacteria were carried out after incubation. If the test showed bacterial contamination, the sample was not used for HYAS activity determination.

According to McCullagh *et al.* (13) the stability of HYAS at this temperature and concentration is very high, and, therefore, a great loss of the enzyme activity during incubation could not be expected. In Table 3 we can see clearly that, during aseptic *in vitro* autolysis of the living tumor, the HYAS activity increased greatly in 7 days. The HYAS activity of the testis also showed a significant increase in 6 days (the half viscosity time fell from 60 to 5 minutes).

DISCUSSION

This study indicates that Walker rat tumor shows HYAS activity and that this activity is not due to bacterial contamination. On the other hand, the Ehrlich mouse carcinoma does not show HYAS activity. The authors cannot, at present, suggest any explanation of this difference.

It was also found that the HYAS activity of testicular and tumor tissue can be raised by sterile necrosis both *in vitro* and *in vivo*. This may be attributed to at least four possible factors: the release of inorganic catalytic agents such as Cu^{++}; the release of nonenzymatic organic oxidative agents such as ascorbic acid; the disappearance of anti-enzymes such as serum antihyaluronidase; and, finally, the liberation of the enzyme from a bound state. The results of the dialysis experiment indicate that substances of low molecular weight are not responsible for the observed depolymerization of hyaluronic acid. Thus, the last two possibilities are left. Perlman, Leonard, and Kurzrock (15) have shown that increased amounts

of HYAS could be obtained from a dying sperm population of rats during incubation of the ejaculate at 37° C. for varying periods. They obtained the same results with incubation of a homogenate of rat testis. Swyer (17) observed increased amounts of enzyme liberated by freezing of the sperm. These observations make it appear probable that the observed increase of HYAS activity of the necrotic tissues can be attributed to the liberation of the enzyme from the bound state during autolysis.

The authors believe that two facts are responsible for the discrepancies in the findings of different investigators on the HYAS content of various human and animal tumors. First, as shown by Kiriluk, Kremen, and Glick (10), bacterial contamination cannot be avoided as a source of HYAS activity in tumors of the skin and mucous membranes. Second, HYAS may be either absent or present in an active or inactive state as a result of the obvious biological differences between various kinds of aseptic tumor tissues.

SUMMARY

The hyaluronidase activity of the Walker rat carcinosarcoma and the Ehrlich mouse carcinoma was determined by the viscometric method. The rat carcinosarcoma showed HYAS activity but in varying amounts in different tumors. Dialysis and heat inactivation experiments showed that the activity was due to enzymes rather than depolymerizing substances of low molecular weight. In all cases the activity was greater in necrotic parts than in living parts of the tissues. Ehrlich carcinoma showed no activity at all. Comparison of HYAS activity in living and necrotic rat testes showed higher activity in necrotic tissue.

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Electrophoretic Analysis of Plasma Protein Constituents in Rats Bearing Regenerating and Pre-neoplastic Livers*

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Concentrations of plasma protein vary during liver damage and regeneration and following evisceration (10, 16). Azo dyes and dietary factors induce liver damage which is followed by the appearance of tumors (2, 7, 12, 17, 18). The protein composition of the liver and, ultimately, the composition of the plasma proteins seem to be affected by such damage (3, 20). Furthermore, a protein-bound dye has been found in the livers of rats fed 4-dimethylaminoazobenzene (DAB) (11), and electrophoretic studies have revealed definite changes in the composition of serum proteins during azo dye carcinogenesis (3). To continue the electrophoretic studies beyond the pre-neoplastic stage and also to test the effects of partial hepatectomy on the electrophoretic patterns, the present study was carried out on plasma from rats on a normal and a deficient diet.

METHODS

Wistar white rats, with an average weight of 150 gm., were placed two in a cage and water was supplied ad libitum. Animals were divided into three groups, of which two were made up of 30 and one of 60 rats. Purina Fox Chow (normal diet) was fed to one group, whereas a second received a basal diet of cooked polished rice plus 1 gm. of carrot per day, and a third group was fed the basal diet in which 4-dimethylaminoazobenzene (concentration, 0.06 per cent) was incorporated by adding the necessary volume of a 3 per cent solution of the dye in cod liver oil. The plasma proteins and the regenerating plasma proteins of the three groups were studied.

Partial hepatectomy was performed according to the method of Higgins and Anderson (6). A mortality of 20 per cent occurred among rats on the basal diet or on the basal plus DAB diet.

Blood samples of 3.0–3.5 ml., collected by cardiac puncture with an oxalated syringe (3 per cent solution of ammonium oxalate) in lightly anesthetized rats, were transferred into oxalated tubes and centrifuged at 2,000 r.p.m. for 15 minutes. Separate animals were used for each cardiac puncture. The plasma samples were removed after centrifugation and diluted with twice their volume of sodium diethylbarbiturate buffer solution of pH 8.3 and ionic strength 0.1. The diluted plasma samples were dialyzed in Nojax cellulose sausage casing bags against 1.2 liters of buffer for a period of 18 hours or more at 5° C.

Electrophoretic measurements were carried out in a Tiselius apparatus with a 2-ml. cell. A current of 5.5 milliamperes was applied, and the bath temperature was maintained at 4° C. Experiments were continued until the fastest component had traveled the length of the cell. Patterns were photographed by the Schlieren scanning method of Longsworth (9) and were divided by the method of Tiselius and Kabat (21). The descending patterns were used for mobility calculations.

Plasma protein concentrations of animals on the diets were studied at intervals of 30 days for a period of 150 days. Studies were carried out at intervals of 24 hours following hepatectomy for a period of 5 days in regenerating liver of rats, whether on the normal diet, deficient diet, or deficient diet with azo dye. The normal diet was fed to all the animals after hepatectomy.

RESULTS

The mobilities of normal plasma proteins, as compared to those of the plasma proteins of rats fed deficient diets, are similar. The mobilities, averaged from sixteen experiments, are as follows: albumin, 6.8; α-globulin, 4.7; β-globulin, 3.8; fibrinogen, 2.5; γ-globulin, 1.1; expressed in units of 10^{-5} cm.$^2 \times$ (v/cm/sec)$^{-1}$. The standard error for all the values is ± 0.2. These values tend to be somewhat higher than the corresponding data reported by some investigators (3, 13).

The percentage values obtained for normal plasma, if recalculated for serum, give the following: albumin, 68; α-globulin, 11; β-globulin, 10;

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γ-globulin, 11. These values agree with those reported by Cook, Griffin, and Luck (3), Deutsch and Goodloe (5), and Moore (13).

The mean percentage composition of rat plasma for different periods, obtained from two to four animals on the basal diet with and without azo dye, are given in Table 1. A decrease in the albumin and an increase in the γ-globulin concentrations occur after 30 days of feeding the basal diet, but these changes diminish progressively with the continued feeding of the basal diet. The other plasma proteins show smaller variations.

A marked difference in the protein concentrations in animals fed the azo dye appears at 60 days, when compared to the protein concentrations for animals on the basal diet. This difference is attributed to the dye. The maximum decrease of albumin and the maximum increase of γ-globulin concentrations are reached at 90 days (Table 1, Chart 1), following which interval the values have a tendency to return to normal levels. However, no variations of the other components are noted, as compared to the basal diet alone, which would indicate that these components are unaffected by the azo dye.

TABLE 1

PERCENTAGE COMPOSITION OF PLASMA PROTEINS DURING CARCINOGENESIS

	Days								
	0	30		60		90		120	150
Diet	Normal	Basal	Basal +DAB	Basal	Basal +DAB	Basal	Basal +DAB	Basal +DAB	Basal +DAB
No. animals	4	2	2	2	4	3	4	4	4
Albumin	53	45	48	49	41	50	38	40	42
α-Globulin	8	11	10	11	12	15	12	11	12
β-Globulin	8	11	12	10	10	12	12	13	12
Fibrinogen	22	19	15	15	17	13	15	17	17
γ-Globulin	9	14	15	15	20	10	23	19	17

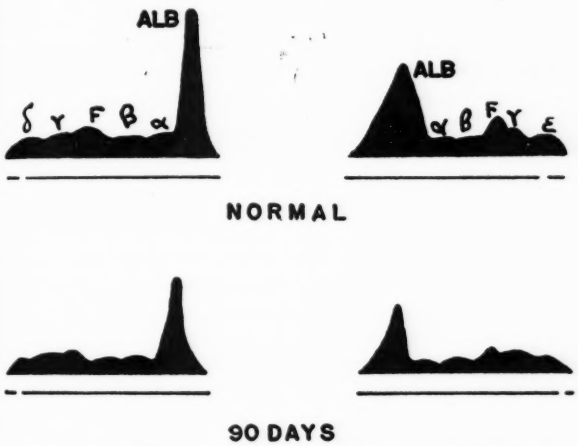

CHART 1.—Electrophoretic patterns of normal rat plasma and of plasma from rat fed the basal diet + DAB for 90 days.

In normal proliferation, as shown in Table 2, variations of albumin and γ-globulin levels occur. A marked effect appears 24 hours after partial hepatectomy and is maintained throughout the period studied. Furthermore, the concentrations of α- and β-globulins increase.

TABLE 2

PERCENTAGE COMPOSITION OF PLASMA PROTEINS IN REGENERATING LIVER*

	Days after hepatectomy					
	0	1	2	3	4	5
No. animals	4	4	4	4	4	4
Albumin	53	39	37	37	37	39
α-Globulin	8	12	15	18	16	16
β-Globulin	8	13	14	14	13	15
Fibrinogen	22	16	15	14	18	13
γ-Globulin	9	20	19	17	16	17

* Rats fed normal diet.

The cumulative effects of cellular proliferation and of azo dye during the combined phenomena of regeneration and carcinogenesis are summarized in Table 3. It would seem that no difference exists in

TABLE 3

PERCENTAGE COMPOSITION OF PLASMA PROTEINS IN RATS BEARING REGENERATING LIVERS, AFTER 55 DAYS ON BASAL DIET OR BASAL DIET PLUS DAB

	Days after hepatectomy						
	0	1		2		3	
Diet	Normal	Basal	Basal +DAB	Basal	Basal +DAB	Basal	Basal +DAB
No. animals	4	3	3	3	3	3	3
Albumin	53	38	37	40	37	39	39
α-Globulin	8	13	15	12	14	13	16
β-Globulin	8	14	14	12	15	12	14
Fibrinogen	22	15	14	16	14	17	14
γ-Globulin	9	20	20	20	20	19	17

the plasma protein composition during the regeneration of liver of rats fed the basal diet or the carcinogenic diet. All plasma components attain similar levels. Furthermore, the variations observed are identical with those occuring during regeneration of normal rat liver.

DISCUSSION

Electrophoretic studies of the plasma proteins during carcinogenesis indicate two distinct features: one, due to the diet alone during the first period of feeding; the other, to the azo dye. The levels of albumin and of γ-globulin fluctuate after feeding the basal diet for a short period, with the protein concentrations returning to normal at 90 days. The differences shown at this period are not statistically significant.[1] During the feeding of DAB, the decrease in albumin and the increase in γ-globulin levels, apparent at 60 days, become statistically significant at 90 days when compared to the albumin and γ-globulin levels for animals on normal or basal diet.[2] These changes, therefore, may be attributed to the dye. The other plasma component levels are unchanged, since they vary very slightly in comparison to the plasma components of rats on the basal diet. The DAB alters only albumin and γ-globulin concentrations. Cook, Griffin, and Luck (3) have reported variations of albumin and γ-globulin concentrations at 4 and 8 weeks for one rat fed 4-dimethylaminoazobenzene, corresponding to those obtained by us at 30 days, which are only due to the basal diet. There is no mention by these authors of values for animals on the basal diet alone. Furthermore, the reported values are normal at 12 weeks, whereas our results show maximum variations at this period. It would seem, therefore, that the variations reported were caused by the basal diet. Their study was pursued during a too short period to allow changes by the azo dye.

Similar variations in albumin and γ-globulin concentrations are observed during liver regeneration, as during carcinogenesis. However, following partial hepatectomy in rats on the normal diet, all the protein levels of plasma vary (α- and β-globulins are affected). The findings confirm and extend for a longer period of regeneration the low albumin value reported for a 2-day period by Roberts and White (17). Their statement indicates that the first protein level to fluctuate, following hepatectomy, is the globulin level, which decreases in the first hours but shows a rapid increase to normal value after 16 hours. Our results indicate that the globulins attain a higher level than the normal value and remain so throughout the 5-day period studied following hepatectomy.

The combined effects of regeneration and carcinogenesis show no differences in results for rats on the basal or the basal diet plus DAB. Regeneration is a process too great in amplitude to be affected significantly by the process of carcinogenesis, and all effects due to the dye may be masked by the rapid cellular proliferation.

The results for normal liver regeneration and liver regeneration in animals fed the basal diet for 55 days indicate no difference. Apparently a diet deficient in protein fed prior to hepatectomy seems to have no effect on the percentage composition of plasma proteins during the proliferation of liver, provided that the animals are put back on a normal diet following hepatectomy. There is no additive effect of diet and the operation.

The formation of tumors in mouse liver by carbon tetrachloride is thought at the present time to be preceded by two steps which are: liver damage and cell proliferation (19). Studies comparing the period of carcinogenesis and the regeneration process indicate that similar changes occur in the albumin and γ-globulin concentrations, but a difference exists in the concentration of the other globulins. During regeneration, the levels of α- and β-globulins increase. They do not vary in carcinogenesis. It would thus seem that the changes occuring in the period of carcinogenesis which has been studied are due to liver damage and not to proliferation, since the two processes may be distinguished by different patterns in quantitative plasma protein composition. Enzymatic studies on depolymerases[3] and on adenosinetriphosphatase[4] carried out in this laboratory led us to consider this period as one of liver damage.

The characteristic variations, in relation to the period of carcinogenesis, appear at 90 days for the plasma protein components. This is the critical period for depolymerase activity (1), as well as for the concentration of ribonucleic acid in hepatic cells (14, 15) and, as this study shows, for the maximum fluctuations of the plasma protein components levels.

[1] For normal compared to basal diet after 90 days feeding: albumin, $t = 0.48$; globulin, $t = 0.61$. These differences are not significant.

[2] For basal diet compared to basal diet plus DAB after 90 days feeding: albumin, $t = 7.70$; globulin, $t = 5.02$. These differences are highly significant.

[3] R. Daoust, G. de Lamirande, and A. Cantero. Nucleodepolymerase Activity in Regenerating Rat Liver (submitted for publication).

[4] C. Allard and A. Cantero, unpublished data.

SUMMARY

Electrophoretic studies of plasma proteins were carried out during carcinogenesis by DAB, regeneration following partial hepatectomy, and during the combined processes of regeneration and carcinogenesis. The following results are noted:

1. In the three conditions there is a decrease in albumin and an increase in γ-globulin concentrations. In addition, however, the α- and β-globulins levels vary during regeneration. This difference in the protein pattern may lead to the conclusion that the period of carcinogenesis which has been studied is a period of liver damage and not of cell proliferation.

2. The results for the combined phenomena of regeneration and carcinogenesis indicate that the effects of regeneration mask the effects of DAB.

3. The maximum variations of albumin and γ-globulin concentrations in plasma occur at about 90 days. Other authors have reported variations in other substances at this period.

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Effects of Ethyl Carbamate (Urethan) on the Early Development of the Teleost *Brachydanio rerio**

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INTRODUCTION

The narcotic urethan (ethyl carbamate) has proved of increasing interest to the cellular physiologist since the first demonstration of its carcinogenic properties (29) in 1943 and the subsequent announcement of its carcinoclastic activities (16) in 1946. It has been shown to arrest or reduce mitotic activity in the eggs of sea urchins (9, 13, 25) and in ciliates (7, 31), as well as in a wide variety of normal and malignant tissue cells both *in vivo* (11, 12, 15) and *in vitro* (3, 14, 18, 26). An initial mitotic stimulatory effect, followed by an inhibition, has been recorded (6, 28) and also a stimulatory effect for malignant cells, in contrast to an inhibition in comparable normal tissues (23).

Since urethan interferes with the process of mitosis, it might be assumed that one of its primary effects on a developing organism would be evidenced as a retardation of growth with a consequent disorganization or disturbance in the normal relationships of embryonic primordia. Concentrations of ethyl urethan above N/30 inhibit cleavage and blastulation in *Rana temporaria*, while concentrations from N/50 to N/100 progressively retard development (32), but no specific effects on differentiation have been recorded. The present investigation was undertaken as another line of approach toward an analysis of the mode of action of urethan as a carcinogenic chemical and mitotic poison, by a demonstration of its effects on the differentiation and organization of embryonic structures.

MATERIALS AND METHODS

Eggs of the zebra fish, *Brachydanio rerio*, a common aquarial cyprinoid, were used as experimental material. These eggs are particularly suitable for observations, since both the chorion (egg membrane) and the early embryos are relatively transparent; the incubation period is of short duration (6–10 days at 25° C.), and spawning is uniform throughout the year.

Eggs in lots not exceeding 50 were reared in finger bowls, incubated at 25° C., and containing 100 cc. of urethan solution in concentrations ranging from 0.05 to 1.25 per cent. Urethan (U.S.P.) was prepared in 5 per cent stock solution in aerated distilled water, from which the experimental range of concentrations was made up by dilution with aquarial water. All solutions were replenished daily.

Six typical developmental stages, namely, early cleavage (1–8-celled blastoderm), blastulation, early to mid-gastrulation ($\frac{1}{4}$–$\frac{1}{2}$ epiboly), closure of the blastopore (termination of gastrulation), optic vesicle, and optic cup were selected as initial periods for subjection to urethan. Exposures were: (*a*) continuous and (*b*) for intervals varying from 4 to 32 hours, followed by transference to fresh aquarial water for subsequent development. The experiments in which exposures were continuous were all run in duplicate, and the results proved to be quite consistent. Differences were no greater than were evident in the controls, when consideration is taken of the fact that variations in susceptibility may occur even among eggs from the same female on successive spawnings.

One experimental series was carried out in which the chorions were pricked with a fine needle prior to exposure to the urethan. There was little or no difference from the controls either within the limits of susceptibility or in the morphological characteristics of the resultant embryos. It was therefore concluded that the chorion is freely permeable to urethan.

EXPERIMENTAL RESULTS

THE EFFECTS OF CONTINUOUS AND SHORT EXPOSURES TO URETHAN ON EARLY DEVELOPMENT OF *Brachydanio rerio*

Urethan exerts a retarding effect on both growth and differentiation of embryonic structures

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in the zebra fish. Its action is proportional both to the duration of exposure and to the concentration. Development was apparently unaltered even by continuous exposure to concentrations of 0.05 and 0.10 per cent, and there was no indication of a stimulatory effect. Concentrations of 0.25 per cent and greater retarded growth and interfered with the orderly processes of differentiation.

Chart 1 is a diagrammatic representation of the general morphological effects resulting from both short and continuous exposures of the eggs to concentrations of 0.10 to 1.00 per cent urethan at the six initial developmental stages, as compiled from all experimental data. Reference to this figure will indicate:

a) Development was normal for all exposures, in concentrations of 0.10 per cent.

b) Development was abnormal following exposures greater than 32 hours in 0.25 per cent.

c) In concentrations of 0.50 per cent, development was abnormal following exposures of 16 hours during cleavage and gastrulation stages, of 32 hours during blastulation, and greater than 32 hours for optic vesicle and optic cup stages.

d) In concentrations of 0.75 per cent, abnormal development resulted when all stages, with the exception of the optic cup, were subjected for periods of 8 hours, and was arrested following exposures of 16 hours for cleavages stages and of 32 hours during blastulation and gastrulation. The optic cup stage exhibited a somewhat lesser susceptibility than did earlier stages, since exposures of 32 hours were required to alter the normal pattern of development.

e) Concentrations of 1.00 per cent completely arrested development for exposures of 4 hours during cleavage and of 8 hours from blastulation to the termination of gastrulation. Development was altered when the latter stages were subjected for a 4-hour period, but only after an exposure of 8 hours during the optic vesicle stage and 32 hours for the optic cup stage.

Concentrations of 1.25 per cent were lethal within 1 day and appeared to inhibit all further development beyond, possibly, the mitoses already in progress, since some cleavages appeared to be completed.

Mortality, in general, was higher for low concentrations where early developmental stages were exposed than for later ones. With short exposures to high concentrations where the normal pattern of development was unaltered, the mortality was correspondingly much lower.

A concentration of 0.25 per cent urethan did not prevent hatching even on continuous exposure, although the percentage hatched was reduced. In general, the shorter the duration of exposure, the greater the numbers proceeding to hatching. For 0.50 per cent urethan hatching occurred following an 8-hour exposure during cleavage, a 16-hour exposure during blastulation, and a 32-hour exposure for the remaining stages, but was completely inhibited on continuous exposures. In 0.75 per cent urethan hatching occurred following a 4-hour exposure during cleavage and gastrulation stages, an 8-hour exposure during blastulation and optic vesicle stages, and up to a 16-hour exposure for the optic cup stage, but in no instance for exposures of 32 hours or greater. For 1.00 per cent urethan hatching occurred following a 4-hour exposure when blastulation stages were initially subjected, an 8-hour exposure for the optic vesicle stages and a 16-hour exposure for the optic cup stages, but was inhibited where either cleavage or gastrulation stages was subjected for even a 4-hour-period.

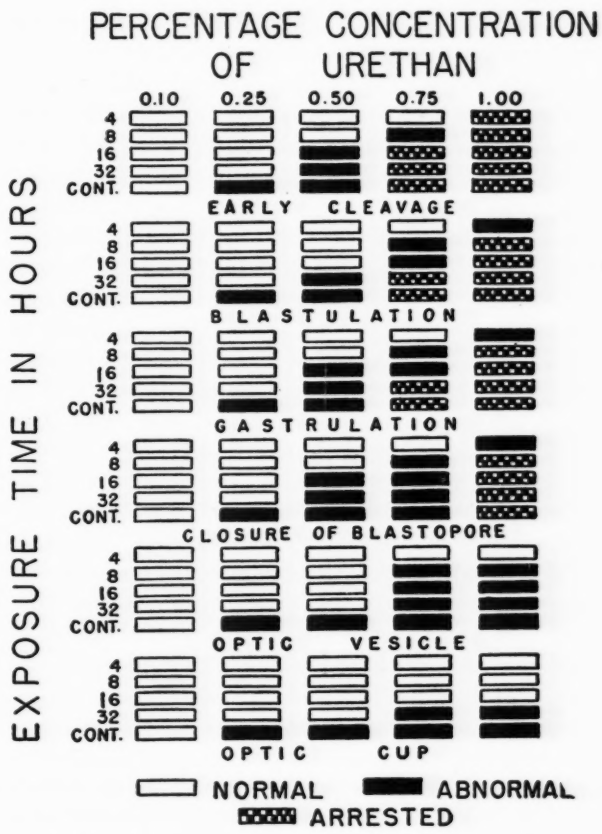

CHART 1.—Diagrammatic representation of the effects of 4-, 8-, 16-, 32-hour and continuous exposures on early development of *Brachydanio rerio* initially subjected to concentrations of 0.10 to 1.00 per cent urethan, during six typical developmental stages: early cleavage, blastulation, gastrulation (¼–½ epiboly), closure of the blastopore (termination of gastrulation), optic vesicle, and optic cup. (3,010 eggs for continuous exposures; 4,150 eggs for exposures of 4–32 hours.)

In teleostean embryos movements of the musculature of the trunk and tail regions and the heart beat offer criteria for estimating metabolic condition. Contractility of the embryonic skeletal muscles does not readily lend itself to quantitative measurements, other than as a determination of the force required to rupture the chorion in hatching, but this force cannot readily be evaluated without consideration of the hatching enzymes (2). Accordingly, the heart rates were recorded as the only readily measurable index of the physiological state of the embryo. The normal rate (176 ± 10 per minute at 25° C.) was unaltered for continuous exposures in concentrations up to 0.25 per cent. At 0.50 per cent there was evidence of a rather sharp decline in rate (65–136 per minute), accompanied by a visible decrease in the regularity, the amplitude, and the force of the beat, which was often entirely inadequate to establish a complete circulation. The heart beat was sometimes initiated in concentrations up to 1.00 per cent when the optic cup stage was attained prior to exposure, but it was invariably slower (less than 136 beats per minute) than normal.

Specific Anomalies Resultant upon Exposure of *Brachydanio rerio* Embryos to Urethan

In general, no particular type of anomaly was induced by urethan. The effects on the morphological organization of the embryo, however, were consistent and reproducible. They were similar to the teratological forms reported by other investigators who employed a variety of physical and chemical agents during the early developmental stages of both teleosts and amphibians. Structural irregularities of the coelom, circulatory system, central nervous system, somatic musculature, sense organs, pigmentation, yolk sac, and epidermis were induced. These abnormalities occurred most frequently where early cleavage to gastrulation stages were subjected to the higher concentrations of urethan employed.

Coelom and circulatory system.—One of the most conspicuous and consistently appearing anomalies was an edema of the coelom similar to that reported for teleostean eggs developing in acidified water (24), and under extremes of temperature and salinity (4) and in x-radiated frog tadpoles (33). In the zebra fish this was apparently initiated in the pericardial cavity (Figs. 1–3) and sometimes spread anteriorly to form subdermal pouches around the eyes. Subsequently, it progressed posteriorly to involve the peritoneal cavity (Fig. 4), causing distention of the body wall ventral and lateral to the viscera and yolk sac.

The heart and extra-embryonic vascular circulation appeared to be especially sensitive to urethan. The former frequently failed to develop its normal flexures and remained as a straight tube with a sparsely developed myocardium. Pressure of the excess fluid in the edematous pericardial cavity may have served to inhibit flexion of the primitive cardiac tube, or flexion may no longer have been necessitated because of the increase in length of the pericardial sac itself. The pericardial edema, combined with the lack of the differentiation of the cardiac tube, resulted in an interrupted and intermittent circulation, which no doubt played an important role in provoking other abnormalities.

Blood corpuscles developed in embryos exposed to concentrations up to 0.75 per cent, but the typical rich vascularization of the yolk sac area was progressively reduced with increasing concentration. Enlarged venous sinuses, congested with erythrocytes, occurred in extra-embryonic areas (Fig. 4) most frequently immediately posterior to the sinus venosus, on the dorsal surface of the yolk sac, and anterior to the anus. These sinuses were often apparently unconnected with adjacent vessels and were no doubt consequent upon incomplete development of the vascular network combined with cardiac insufficiency. They were comparable to those which appear in metrazol-treated embryos of *Fundulus heteroclitus* (30).

Central nervous system.—The central nervous system exhibited a variable response to urethan. On continuous exposure to concentrations of 0.75 per cent, development was inhibited more especially in the head region, where practically no differentiation of the brain occurred (Fig. 2), and the spinal cord was sometimes shortened or deformed. For shorter exposures, parts of the brain appeared to be enlarged and malformed. Regional duplication of the central nervous system occurred in varying degrees and was accompanied by a corresponding duplication of the notochord.

The arrangement of cells within the central nervous system was often unusual, the typical cellular pattern especially in the spinal cord being disrupted (Fig. 5). Two types of cells were present, one deeply staining and generally more peripheral in location, the other with a lighter staining reaction and more central in position. It has been suggested by Dr. M. L. Barr[1] that the latter type may represent less differentiated cells. A diminishing staining reaction and an actual breakdown of the internal layers of the nervous system have been shown in teleostean embryos to follow metrazol

[1] Personal communication, Dr. M. L. Barr, Professor of Anatomy, Medical School, University of Western Ontario, London, Canada.

treatment (30). Irregularities in the cellular organization of the nervous system of embryos of Fundulus also occur following subjection to alcohol (39), and of *Rana pipiens* treated with 2,4-dinitrophenol (10). Progressive degeneration of the neural fold tissue of the latter species also is consequent upon administration of DBAS (5).

Occasionally, vacuolated areas appeared in the spinal cord of the urethan-treated zebra fish (Fig. 6) presumably associated with edema and with hydropic degeneration of the adjacent musculature.

Body axis.—Deformities of the body axis involving both the notochord and musculature were common, especially when the embryos were subjected to the higher concentrations. These corresponded to abnormalities reported in teleosts by other investigators as a resultant of abnormal temperatures (19, 34), various metallic salts, and reduced oxygen supply (37, 38, 40), products of metabolism such as urea (43), ultraviolet light (17), 2,4-dinitrophenol (41), and x-rays (36).

A reduction in the numbers of myotomes in the zebra fish embryo resulted in a shortening of the body axis. Curious flexures of the tail, which became bent at various angles, were sometimes attributable to an unusual arrangement of the musculature, such as unilateral absence or unequal lateral distribution. Some embryos with both musculature and notochord apparently normal showed pronounced flexures of the trunk and tail regions which could only be attributed to localized tetanic contractions resembling those reported for metrazol-treated *Fundulus heteroclitus* (30).

Hydropic degeneration of the musculature was of frequent occurrence (Fig. 6) and involved a greater or lesser portion of the myotomes. In some instances the fibers lacked orientation (Fig. 7), when contrasted to the characteristic longitudinal arrangement in the trunk and tail regions of normal embryos. Some fibers radiated laterally from the notochord and were intermingled with longitudinally disposed ones, while others were enlarged and possessed vesicular nuclei and only sparsely scattered myofibrils.

Sense organs.—Deviations from the normal with respect to eye formation appeared especially when gastrulation stages were subjected to urethan for short periods, and in no instance where the initial exposure followed closure of the blastopore. The eyes sometimes failed to develop, were abnormally small (Fig. 2), or exhibited partial to complete median fusion. Adelmann (1) has summarized the reported chemical agents which induce similar cyclopean eyes. The types produced in urethan-treated zebra eggs are comparable to those recently recorded in teleosts following subjection to 2,4-dinitrophenol (41) and colchicine (42).

Pigmentation.—In almost all terata there was a tendency to deviate from the normal distributional pattern of melanophores of both the eyes and body surface. Following continuous exposure to concentrations near the upper limits of tolerance, pigmentation was absent or sparse (Fig. 2). Shorter exposures resulted in irregularity in melanophore distribution, including localized massive accumulations. In high concentrations, xanthophore production appeared to be retarded and often absent.

Yolk sac.—Following exposure to urethan, the yolk sac became distorted and lateral diverticula appeared (Fig. 2) at one or more regions on the surface. Occasionally, a posterior ventral diverticulum in the mid-line would seem to be indicative of a persistent blastopore corresponding to those in 2,4-dinitrophenol-treated amphibian (10) and teleostean embryos (41). When coelomic edema was excessive, the yolk presented a coarsely granular appearance which might be interpreted as a separation of the granules by fluid.

Epidermis.—Epithelial hyperplasia occurred in localized areas of the epidermis of urethan-treated embryos. The most characteristic proliferation was initially visible in the living embryo over the anterior or ventral surface of the pericardial sac (Fig. 1). Later, similar hyperplastic activity of the epidermis was apparent on the ventro-lateral trunk regions and occasionally on the tail (Fig. 2).

The epidermis of the normal embryo is of a stratified squamous type (Fig. 8), two to three cells in depth, and with a total thickness of 10–15 μ. The basal cells are of a low cuboidal character, while those of the superficial layers are slightly more flattened. The cytoplasm is finely granular and shows no indication of superficial cornification.

In epidermal areas exhibiting hyperplasia (Fig. 9) the epithelium varied in thickness from 20 to 70 μ. Many of the superficial cells and a few of the deeper ones were flask-shaped (Fig. 10) with bulbous distal portions varying from 10 to 15 μ in diameter. The narrow elongated neck extended toward the basement membrane. The cytoplasm was coarsely granular and frequently contained enlarged vacuoles proximal to the nucleus and extending into the neck region. A high proportion of the proliferating epithelial cells were spherical, with diameters ranging from 15 to 25 μ. They possessed one or two clear central vacuoles (Fig. 11) and usually several smaller superficial ones. Coarsely granular masses, presumably representing inclusions resultant upon phagocytic activity,

or central areas of necrosis were often present in the vacuoles or throughout the cytoplasm. They stained densely with iron hematoxylin but reacted negatively to Feulgen's stain. Because of the vacuolization of the cytoplasm the nucleus was crowded into a superficial position and sometimes became crescentic in outline. Multinucleate cells possessing large vacuoles were of frequent occurrence (Fig. 12) and ranged in diameter from 25 to 45 μ.

DISCUSSION

The resistance of the embryos of *Brachydanio rerio* to urethan has been shown to vary during development. The earlier the stage subjected, the more marked the retardation in development, and the more pronounced is the degree of abnormality induced. As a corollary, the later the stage exposed, the greater its resistance, and the lesser the tendency toward deviations from normal development. The effects may be considered to be cumulative and, in general, proportional to the concentration and to the duration of exposure.

Although there was a progressive decrease in the relative sensitivity from early cleavage to the optic cup stage, the anomalies induced in concentrations from 0.25 to 0.75 per cent followed a typical pattern. Stages up to and including gastrulation, however, were exceptional, since, in addition to the abnormalities characteristically appearing on subjection of other stages, structural defects of the eye were common. The abnormalities induced were in most instances comparable to these reported in the literature as a resultant of the action of a wide variety of chemical and physical agents on developing eggs of other species of both teleosts and of amphibians.

The congestion of blood corpuscles in venous sinuses in the extra-embryonic areas may be attributable to a direct effect of the urethan. A dilation of the capillaries and an increase in their permeability proceeding to stasis follow applications of urethan to the surface of the frog tongue (21). A similar effect in mesenteric capillaries of the frog (22) has been attributed to direct toxicity of the urethan on the endothelium, combined with increased pressure due to blocking of the venous capillaries. It has also been shown to induce glomerular lesions in mice (20) and edema resultant upon capillary damage in the lungs of leukemic mice (44). Capillaries are incapable of proliferating in the presence of "mitotic arresting" doses of urethan in the albino rat (27). It is therefore conceivable that the areas of extra-embryonic vascular congestion, as well as the coelomic edema induced in the zebra fish by urethan, may be partially correlated with a plasma loss consequent upon endothelial damage combined with inhibition of capillary development.

The epithelial hyperplasia of the epidermis induced by urethan is comparable to certain epithelial tumors reported for various teleosts in nature (35). Many of these, however, involved papillary proliferation of the connective tissue of the corium, which was not evident in Brachydanio. Papilliform outgrowths or epithelioma have been recorded in 2,4-dinitrophenol-treated frog embryos (10).

The effects of urethan as described here offer an interesting comparison with the results of Briggs and Briggs (5), who subjected the eggs of *Rana pipiens* to the water-soluble carcinogen Na-1,2,5,6-dibenzanthracene-9,10-endo-α,β-succinate (DBAS). They found no evidence of a stimulative effect in any concentration, a retardation of developmental rate in proportion to concentration, a uniform pattern of degeneration in strong concentrations, and a differential retardation of the capacity for muscular movement but no alteration of the morphological organization of the embryos. Cate (8), however, observed a marked acceleration in growth of the larvae of *Rana esculenta* in low concentrations of DBAS and a retardation at high concentrations. There was no evidence of growth stimulation by urethan at any of the concentrations used in this investigation, but Laing[2] noted an accelerated growth of the embryonic axis of the teleost, *Salmo trutta fario* L., in proportion to concentration in solutions of 0.10 and 0.25 per cent urethan. The retardation of developmental rate by low concentrations of urethan and inhibition by higher concentrations, as demonstrated for embryos of Brachydanio, correspond to those of Parnas and Krasinska (32) for *Rana temporaria* and are in agreement with the findings of many investigators for mitosis of egg cells, ciliates, and various tissue culture preparations.

Embryos of Brachydanio reared in concentrations of 0.50–0.75 per cent urethan exhibited disorganization and apparent lack of differentiation of both nervous and muscular tissue. This may be indicative of a differential retardation of development or of a degeneration corresponding to that initiated during an earlier stage in DBAS-treated frog embryos (5). An inhibition of the capacity for muscular movement following subjection to urethan is evidenced by the fact that even where the musculature was apparently normal the embryos were unable to hatch following continuous exposure to concentrations of 0.50 per cent.

[2] M. A. Laing, The Effect of Ethyl Carbamate (Urethane) on the Egg of the Brown Trout (*Salmo trutta fario* L.). Unpublished M.Sc. thesis, Department of Zoology, University of Western Ontario, 1950.

The effects of urethan on the developing zebra fish are thus, in certain respects, comparable to those of DBAS on the frog embryo, but differ in that the morphological organization of the embryo has been modified. The anomalies resultant upon subjection to urethan have been shown to be of the types which might be consequent to the application of any extreme physical and chemical factors which disturb the orderly processes of growth and differentiation. On this basis, such terata cannot be attributed to specific properties of the chemical compound. The vascular stasis in the extra-embryonic area, and more especially the induction of epithelial hyperplasia of the epidermis, nevertheless, are suggestive of specific effects, and subsequent investigation is being directed along these lines.

SUMMARY

1. Six typical developmental stages of the teleost, *Brachydanio rerio*, namely: early cleavage, blastulation, early to mid-gastrulation, late gastrulation (closure of the blastopore), optic vesicle, and optic cup were subjected to concentrations of 0.05–1.25 per cent urethan (ethyl carbamate) for periods varying from 4 to 32 hours and continuously.

2. The chorion is freely permeable to urethan, as evidenced by the fact that no significant differences in susceptibility occur when it is pricked.

3. Retardation of both growth and differentiation of embryonic structures occurs in concentrations of 0.25 to 1.25 per cent and results in the production of anomalies.

4. The effects are cumulative and proportional to the concentration, the duration of exposure, and to some extent the initial stage of development on subjection. The sensitivity, in general, decreases with age and differentiation.

5. No special type of teratological embryo is exhibited, but the results are consistent and reproducible. Developmental abnormalities include: edema of the coelomic cavities and hydropic degeneration of various body tissues, cardiac and systemic circulatory irregularities, disorganization and lack of differentiation of the central nervous system and the eye, malformation of the notochord and musculature, atypical pigmentation, and epithelial hyperplasia.

6. Vascular stasis in the extra-embryonic area and, more especially, the occurrence of extensive epithelial hyperplasia of the epidermis may be indicative of specific effects of the urethan, while the other anomalies resemble types resultant upon the application of a variety of physical and chemical agents to the developing embryo.

ACKNOWLEDGMENTS

The authors wish to express their indebtedness to Dr. M. L. Barr and Dr. J. C. Paterson, Faculty of Medicine, University of Western Ontario, for examination of microscopic preparations, to Mr. Mont Moore, Port Stanley, Ontario, for the supply of adult zebra fish, and to Mr. C. E. Jarvis for the preparation of photomicrographs.

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Figs. 1–5.—Urethan-treated embryos of the zebra fish (*Brachydanio rerio*).

Fig. 1.—Camera lucida drawing of embryo resultant upon initial subjection during early cleavage to 0.75 per cent urethan for 8 hours and subsequent transference to aquarial water for 3 days. Note edema of pericardial cavity and expanded melanophores. Mag. ×40.

Fig. 2.—Camera lucida drawing of embryo of 3 days resultant upon continuous exposure to 0.75 per cent urethan from late gastrulation. Note slender contorted embryonic axis, reduced pigmentation, small eyes, coarsely vesicular yolk, and epithelial hyperplasia over the pericardial sac and caudal fin regions. Mag. ×40.

Fig. 3.—Transverse section (10 μ) at the level of the pericardial cavity and otic vesicle, of a 6-day embryo, following continuous exposure to 0.50 per cent urethan from the optic cup stage. The distension of the pericardial cavity and the subdermal spaces dorsal to the rhombencephelon indicate extreme edema. The unflexed cardiac tube appears as two concentric cellular rings, representing the endocardium and reduced epimyocardium. Mag. ×117.

Fig. 4.—Transverse section (10 μ) at the mid-level of the liver of an embryo resultant upon initial subjection during the optic vesicle stage to 0.75 per cent urethan for 8 hours and subsequent transference to aquarial water for 6.5 days. Note the venous sinus massed with erythrocytes ventral to the liver and extreme edema of the coelomic cavity. Mg. ×117.

Fig. 5.—Transverse section (6 μ) at the posterior level of the pectoral fins of a 5-day embryo following continuous exposure to 0.50 per cent urethan from gastrulation. Note the reduced musculature and few myofibrils probably indicative of hydropic degeneration. Two notochords are present at this level. The arrangement of the neurones in the spinal cord is atypical, the central ones staining more lightly and appearing to be less differentiated than the more peripheral ones. Mag. ×450.

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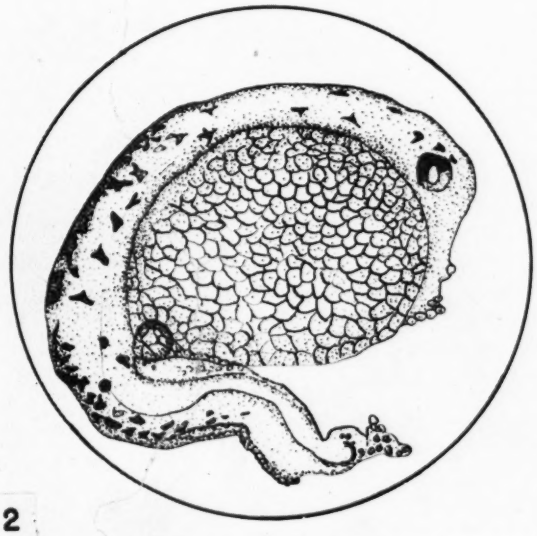

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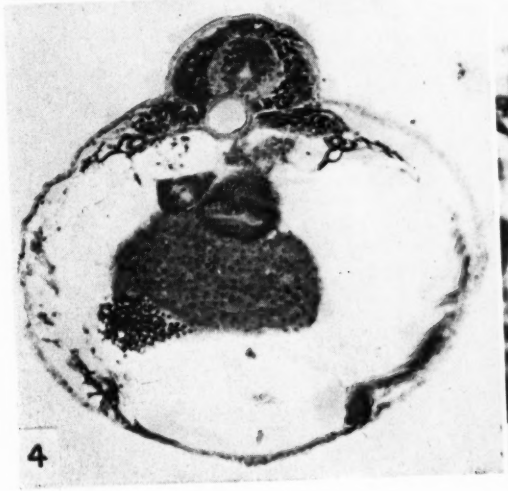

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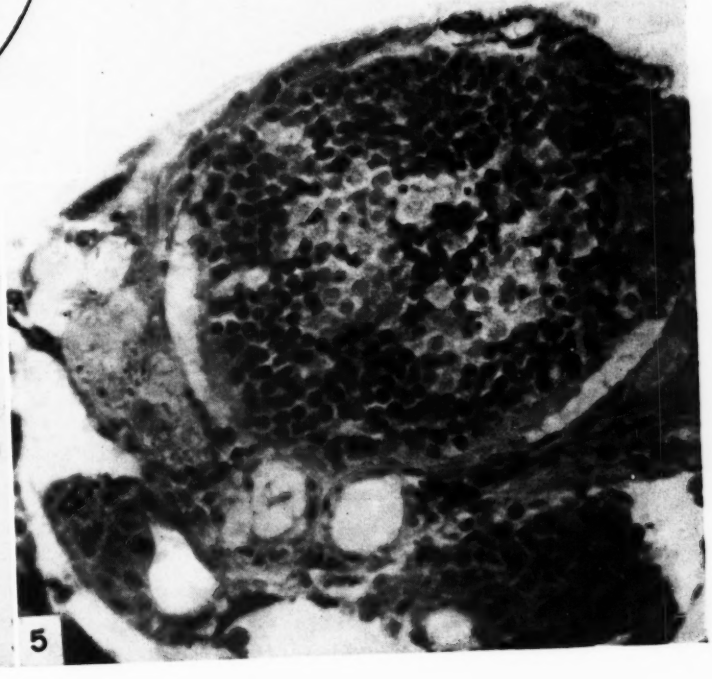

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FIGS. 6–12.—Urethan-treated embryos of the zebra fish (*Brachydanic rerio*).

FIG. 6.—Transverse section (10 μ) through the mid-trunk region of a 6-day embryo following continuous exposure to 0.50 per cent urethan from the optic vesicle stage. The myotomes of the left side are composed of irregularly scattered fibers, the limits of which are difficult to define. Hydropic degeneration is indicated. A large vascuole is present in the right ventral region of the spinal cord. Mag. ×450.

FIG. 7.—Transverse section (10 μ) through the mid-body region of a 5-day embryo following continuous exposure to 0.50 per cent urethan from gastrulation. Note the irregularly arranged musculature; some fibers radiate laterally from the double notochord, while others which parallel the long axis of the body are sectioned transversely. The nuclei of the latter fibers are vesicular and the myofibrils sparse. Hydropic degeneration is indicated. Mag. ×675.

FIG. 8.—Transverse section (10 μ) through the epidermis of a control embryo of 5 days, adjacent to the yolk sac. The surface epithelial cells are squamous in type, while the basal ones are low cubodial. Mag. ×675.

FIG. 9.—Transverse section (6 μ) through the epidermis surrounding the yolk sac of a 5-day embryo following continuous exposure to 0.50 per cent urethan from gastrulation. Epithelial hyperplasia is indicated. Mag. ×675.

FIG. 10.—Transverse section (6 μ) through the epidermis of the embryo illustrated in Figure 9. The superficial cells of this hyperplastic epithelium are flask-shaped, and many possess cytoplasmic vacuoles which may appear transparent or contain coarsely granular material. Mag. ×1,450.

FIG. 11.—Transverse section (10 μ) through the epidermis surrounding the yolk sac of a 5-day embryo following continuous exposure to 0.50 per cent urethan from gastrulation. Note the peripheral nuclei and evidences of phagocytosis. Mag. ×1,450.

FIG. 12.—Transverse section (10 μ) through the epidermis surrounding the yolk sac of a 3-day embryo following continuous exposure to 0.75 per cent urethan from the termination of gastrulation. Note the giant cell possessing three nuclei and two vacuoles. Mag. ×1,450.


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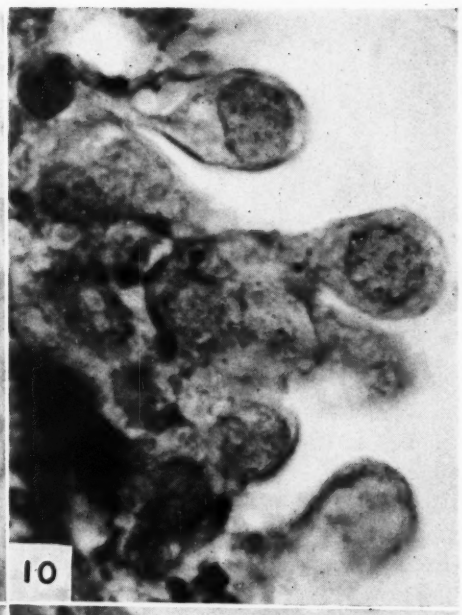
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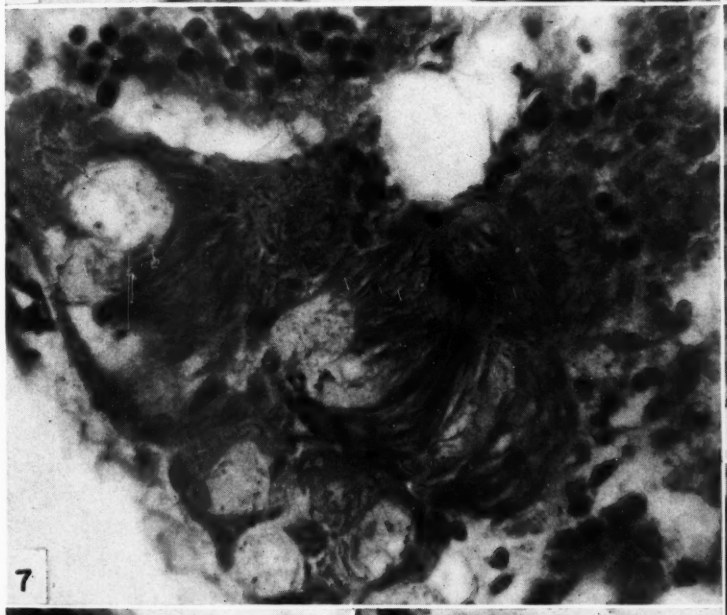
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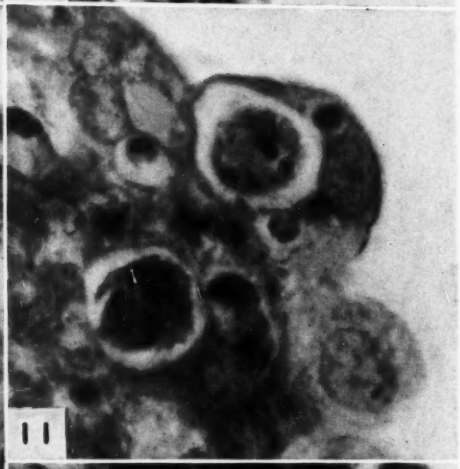
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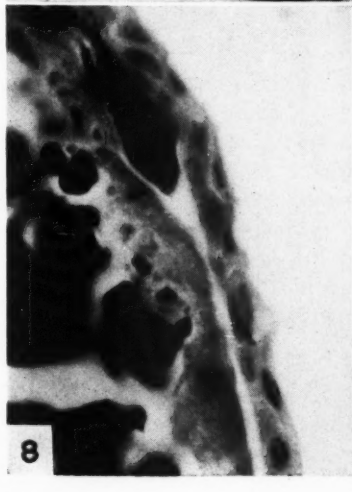
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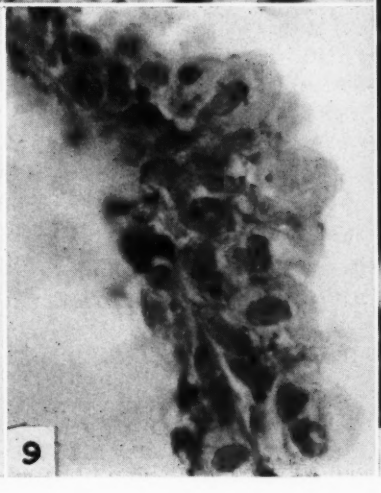
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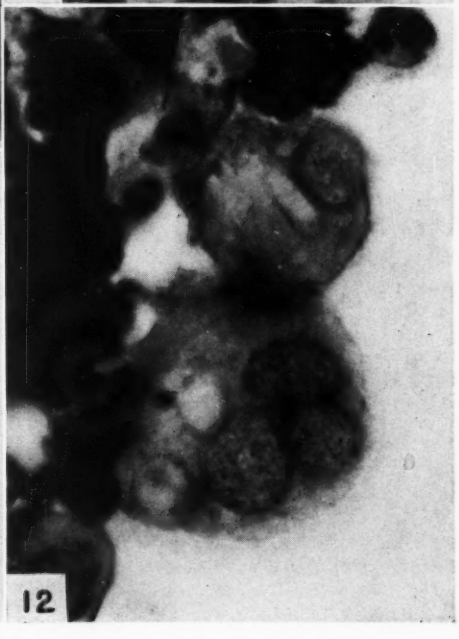
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The Viscosimetric Determination of Desoxyribonuclease Inhibition*

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Studies are to be reported on a naturally occurring inhibitor of desoxyribonuclease. The present paper deals with the technic for its determination.

MATERIALS AND METHODS

Substrate.—Desoxyribonucleic acid (DNA) was prepared from beef thymus according to Mirsky and Pollister (8) and McCarty (7), except that no attempt was made to remove all traces of histone. Later, the method of Marko and Butler (6) was used, which yields a product of high purity with a minimum of effort. It was dissolved in either water or 0.03 M veronal buffer, pH 7.3–7.5, as desired, and the concentration adjusted, after dilution, to yield a final relative viscosity of 2.

Enzyme.—Crystalline desoxyribonuclease,[1] approximately 1.3 μg. per milliliter, was dissolved in a solution containing 0.225 M magnesium sulfate and 0.25 per cent gelatin. The enzyme concentration was adjusted so as to cause a reduction in the relative viscosity of the substrate to ½ its initial value (half-viscosity time) in between 90 and 120 seconds.

Cell extracts.—Extracts of whole blood (sometimes heparinized) or bone marrow (secured by needle puncture of the ilium) were prepared by dilution with neutral water (1/100). A cell count was made prior to dilution. The extracts were centrifuged clear and placed on ice until used. The inhibitory activity of whole blood is attributable to the white blood cells. Neither hemolyzed red blood cells nor heparin has any inhibitory activity. Serum contains an insignificant amount of inhibitor, which probably arises from disintegrated white cells (see below).

Two ml. of substrate-buffer solution was mixed with 0.2 ml. of enzyme solution and 0.5 ml. of water. In some experiments, the substrate was dissolved in water and the buffer was added to the enzyme prior to mixing of the two. In measuring the inhibitory effect of the cell extracts, a mixture of cell extract, enzyme, and water was incubated for 5–10 minutes at 25° C. prior to mixing with the substrate. The enzymatic depolymerization of the substrate with time was then measured in a 30-second Ostwald viscometer at 25° C. The relative viscosities of the media at various times were plotted, and the time required to reach ½ the initial viscosity (half-viscosity time) was determined by graphic interpolation in accordance with the method of Haas (2) for the determination of hyaluronidase activity (Chart 1).

Kinetics.—The half-viscosity time is inversely proportional to the enzyme concentration and directly proportional to the concentration of the inhibitor (i.e., the amount of enzyme neutralized by the cell extract) within limits (Chart 2). The inhibition of the enzyme is quantitative irrespective of enzyme concentration (Table 1).

The relationship between the cell concentration (cell extract) and the percentage inhibition is more complex (Chart 2). Between 15 per cent and 35 per cent inhibition the relationship is approximately linear. In the lower range of cell concentrations, the nature of the curve suggests the presence of enzyme in the cell extract, which is probably released from combination with the inhibitor by dilution. The existence of desoxyribonuclease in mammalian cells is, of course, well established but was not previously demonstrated in this manner. As a consequence, it is evident that this technic determines not the absolute amount of inhibitory activity but rather the excess of inhibitor over and above that neutralized by the naturally occurring enzyme in the cell extract.

From the technical point of view, it is essential that the concentration of cell extract be such as to yield an inhibition below 60 per cent and above 10 per cent (or be considerably greater than that from 10^4 cells). In the determination of the inhibitory activity of human white blood cells, the extract of 10^4 cells per test was commonly used. In the measurement of bone marrow and other

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[1] Crystalline desoxyribonuclease was obtained from Worthington Chemical Corp., Freehold, N.J.

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tissue cell inhibition, 5×10^4 to 20×10^4 cells were used.

The results are expressed as the percentage inhibition per 10^4 cells (see below), and the curve (Chart 2) is used as a calibration curve. Below 10 per cent the curve is taken to be linear and to go through zero inhibition.

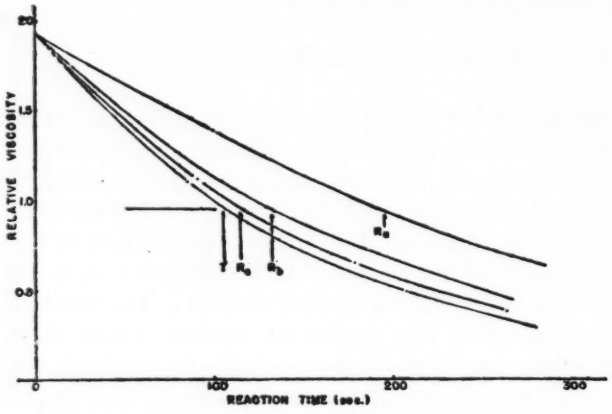

CHART 1.—Changes in viscosity of DNA solutions containing a constant amount of enzyme and varying amounts of inhibitor.

T = Half-viscosity time of mixture: 2.0 ml. substrate-buffer +2.0 ml. enzyme +0.5 ml. water.

R_a = Half-viscosity time of mixture: 2.0 ml. substrate-buffer +0.2 ml. enzyme +0.5 ml. rat blood (diluted 1/1000 with water).

R_b = Same, except 0.2 ml. of rat blood solution and 0.3 ml. water used.

R_c = Same, except 0.1 ml. rat blood solution and 0.4 ml. water used.

Example: Flow time of buffers = 37.5 sec.: of substrate without enzyme = 109.5 sec. η_r of substrate $= \frac{109.5}{37.5} - 1 = 1.92$.

T time* (sec.)	T flow time (sec.)	T reaction time (sec.)	η_r†	R_a time (sec.)	R_a flow time (sec.)	R_a reaction time (sec.)	η_r†
		0	1.92			0	1.92
47	78.7	86	1.10	42	90.3	87	1.41
180	55.8	208	0.49	153	73.7	190	0.965
287	48.8	281	0.30	253	61.4	284	0.64

Calc. T = 105 sec. R_a = 192 sec.

$$\text{Per cent inhibition} = \left(\frac{1}{T} - \frac{1}{R_a}\right) 10{,}000.$$

$$= (0.00952 - 0.00521)\ 10{,}000 = 43.1 \text{ per cent.}$$

* After addition of enzyme

† Relative viscosity.

Calculation of the percentage inhibition.—In order that results may be comparable, the percentage inhibition was calculated on the basis of an enzyme concentration of 100 seconds half-viscosity time. Advantage was taken of the facts that the enzyme concentration is inversely proportional to the half-viscosity time and that the amount of enzyme destroyed is quantitatively related to the amount of inhibitor.

$$\text{Absolute amount of enzyme neutralized} = \frac{1}{T_a} - \frac{1}{R_a} = \frac{1}{T_o} - \frac{1}{R_o}.$$

T_a = observed half-viscosity time of enzyme prior to inhibition (sec).

R_a = observed half-viscosity time of enzyme after inhibition (sec).

T_o = 100 seconds.

R_o = half-viscosity time of R, when T is 100 seconds.

$$\frac{1}{R_o} = \frac{1}{T_o} - \frac{1}{T_a} + \frac{1}{R_a} \qquad (a)$$

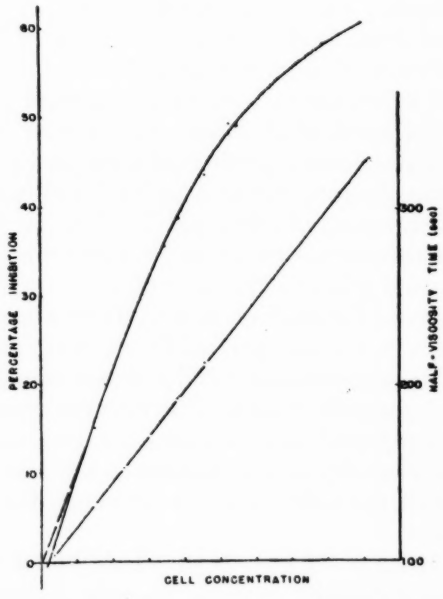

CHART 2.—Relationships between cell concentration and (*a*) half-viscosity time and (*b*) percentage inhibition.

The right hand curve shows the relationships between the cell concentration and the half-viscosity time.

The left hand curve indicates the relationship between the cell concentration and the percentage inhibition. Both curves were constructed from the same data of a number of experiments.

TABLE 1

RELATIONSHIP OF ENZYME INHIBITION TO ENZYME CONCENTRATION
(Inhibitor Concentration Constant)

Enzyme per test (μg.)	Inhibition (per cent)	Enzyme inhibited (μg.)
0.376	22.3	0.0838
0.286	34.3	0.0981
0.188	47.7	0.0897
		0.0905 (mean)

$$\text{Fraction of enzyme neutralized } (T_o = 100 \text{ seconds.}) = \frac{\frac{1}{T_o} - \frac{1}{R_o}}{\frac{1}{T_o}}. \quad (b)$$

Substituting (a) for $\frac{1}{R_o}$ in (b):

$$= \frac{\frac{1}{T_o} - \left(\frac{1}{T_o} - \frac{1}{T_a} + \frac{1}{R_a}\right)}{\frac{1}{T_o}} = \frac{\frac{1}{T_a} - \frac{1}{R_a}}{\frac{1}{T_o}} = \left(\frac{1}{T_a} - \frac{1}{R_a}\right) \times 100.$$

$$\text{Percentage of enzyme neutralized (i.e. inhibition)} = \left(\frac{1}{T_a} - \frac{1}{R_a}\right) \times 10{,}000.$$

released and by the change in optical density caused by the action of the enzyme on the substrate.

An identical curve with an optimum in the neighborhood of pH 6.75 is obtained for the inhibitory action of cell extracts on the enzyme (Chart 3). In this instance, the enzyme concentrations were varied to give the same half-viscosity time of approximately 100 seconds at each pH. Unexplained is the fact that at both extremes of pH, not only is the inhibitory effect of the cell extract abolished, but there is an increase in

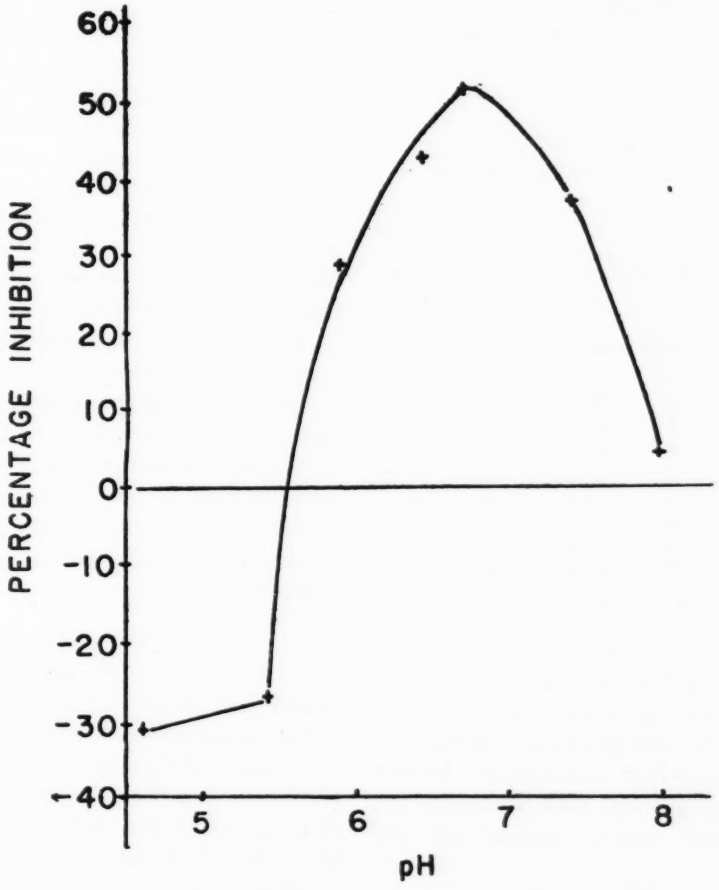

CHART 3.—The effect of hydrogen-ion concentration on the inhibitory activity of cell extracts

RESULTS

Effect of hydrogen-ion concentration and ionic strength on enzyme activity and on inhibitor activity.—In confirmation of Laskowski and Seidel (5) and Kunitz (4), the enzyme activity was found to have an optimum pH between 6.75 and 7.0. The former workers measured the enzyme activity by its viscosity-reducing action and the production of acid-soluble products; the latter measured it by means of the acid-soluble products enzyme activity (indicated as a negative percentage inhibition) over and above that found in the absence of cell extract. This is probably due to a protective effect of some cell components on the enzyme, rather than to the additional desoxyribonuclease in the cell extract.

Increasing the amount of veronal buffer causes a decrease in the activity of the enzyme at any pH but has little or no effect on the activity of the inhibitor.

Effect of hydrogen-ion concentration on the inhibitor content of cell extracts.—The effects of hydrogen-ion concentration on the reaction between the desoxyribonuclease and its inhibitor raises the question whether the inhibitor is destroyed or simply inhibited. Cell extracts were made up in water of either pH 5 or pH 7, then buffered by acetate-veronal buffer to a pH of 5.92 or 7.45, respectively, and permitted to stand for variable periods prior to testing. In no instance was a significant change in the amount of inhibition noted. In addition, in the course of routine tests, the cell extract-enzyme mixture was subjected to a pH of 5.69, due to the magnesium sulfate, or buffered to a pH of 7.35. In neither case were differences in inhibition noted. Hence, it is evident that the pH of the solution has no effect on either the enzyme or the inhibitor but simply prevents their reaction. It is also evident that, since no interaction occurs at the acid pH values (Chart 3), the reaction of the inhibitor and enzyme at the optimum pH is instantaneous, and reversible, as claimed for the yeast desoxyribonuclease inhibitor (10).

TABLE 2

EFFECT OF TIME ON THE INHIBITOR ACTIVITY OF BLOOD AND BONE MARROW CELL EXTRACTS

		Blood		Bone Marrow	
Subj.	Disease	Hours*	Per cent inhibition	Hours*	Per cent inhibition
G. S.	Normal	½	38.5	½	45.9
		1½	36.9	2½	44.8
		3½	44.8	4	35.2
G. D.	Chronic myelogenous leukemia	1	60.4	½	54.3
		3	59.4	1½	54.5
		3½	55.9	3½	58.3
		4	60.4		
H. H.	Chronic myelogenous leukemia	2½	38.8	2½	17.6
		4	44.8	3½	16.6
		5	43.4	4½	16.6
		6½	33.6	5	12.8
		7	42.9	6	15.1

* Time after the cells were taken and diluted with water.

Effect of time on the inhibitor content of blood and marrow cell extracts.—Of particular importance to the determination of the inhibitory activity of cell extracts is the stability of the inhibitor during the time required to perform the tests. Cooper *et al.* (1) have shown that the inhibitor content of normal lymph node, lymph node metastasis, and cancer of the breast disappears in a day. We have also found that cell extracts of liver and spleen (rat) manifest a complete or nearly complete loss of their inhibitory activity in 24 hours. Normal white blood cells, when exposed to the procedure essential to the preparation of commercial plasma[2] (15–20 hours in the cold), suffer a total loss of inhibitor.

However, water extracts of human blood and marrow cells kept on ice show no significant loss of inhibition over a period of from 4 to 7 hours (Table 2). In this same period extracts of rat liver, spleen, the Gardner lymphosarcoma, and the Murphy tumor all show considerable losses of inhibitor.[3] Hence, a decided difference exists between the rate of disappearance of the inhibitor from white blood and marrow cells and from organ tissue cells. This difference is due, at least in part, to the protective action of plasma.[3]

The inhibitor content of human serum.—In twelve persons, nine normal and three with various types of cancer, the inhibitor content of the serum was found to vary between 0 and 0.8 per cent per 0.001 ml. In two instances no inhibition but desoxyribonuclease activity was found. The mean was approximately 0.4 per cent per 0.001 ml. This amount of inhibition can be easily accounted for by the break-up of about 100 white blood cells, since 10,000 white blood cells have an inhibitory value of 37.4 per cent (3).

Wróblewski and Bodansky (9) have noted the occurrence of enzyme in human serum. Their technic required the incubation of the serum for a number of hours, which could account for the loss of enzyme inhibitor. Their results and ours would indicate that a small amount of enzyme does exist in serum, masked, usually, by an excess of inhibitor, and its origin would appear to be the white blood cells.

SUMMARY AND CONCLUSIONS

1. A viscosimetric method is described for the determination of desoxyribonuclease activity and of the desoxyribonuclease inhibitor.

2. The inhibitory activity of cell extracts is optimum at pH 6.75. At acid pH values, interaction between the inhibitor and the enzyme is prevented, but the inhibitor is not destroyed. The reaction between the inhibitor and enzyme is instantaneous and reversible.

3. Extracts of human white blood cells and bone marrow cells, in the presence of plasma, show no significant losses of inhibitor activity over a 4–7-hour period, but gradually lose their activity over a period of 24 hours or more.

4. The inhibitor content of human serum is low, about 0.4 per cent/0.001 ml, and probably arises from the white blood cells.

[2] Furnished through the courtesy of the Hyland Laboratories, Los Angeles, Calif.

[3] To be reported.

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An Inhibitor of Desoxyribonuclease in Human White Blood and Bone Marrow Cells, and Its Relationship to Cellular Maturity*

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It has been postulated that control or inhibitory mechanisms are important aspects of vital cellular functions (3, 10). Since enzymatic reactions are basic to cellular metabolism, the inhibition of enzymatic activity probably plays a fundamental role in the regulation of life processes. Comparative studies of malignant and normal cells have also led to the conclusion that the comparable processes in the normal cell are under the control of inhibitory factors (2, 3, 8, 10). Therefore, because of the key position of desoxyribonucleic acid in the cell, the existence of a specific inhibitor of desoxyribonuclease and its variation under different conditions of cellular activity would be of considerable interest.

While inhibitors of desoxyribonuclease have been described in yeast (12, 13), in serum (9), and in various mammalian tissues (4, 5), no studies have been reported on the inhibitor in human normal and leukemic white blood and bone marrow cells with which this paper is concerned. The present studies suggest that there exists a relationship between the degree of cellular maturity and the amount of inhibitory activity per cell.

METHOD

The viscosimetric technic used for the determination of the desoxyribonuclease inhibitor (anti-DN-ase) has been described (7). The results are expressed as the percentage inhibition per 10^4 cells, as referred to a constant amount of enzyme activity.

RESULTS

Normal human white blood and bone marrow cells.—The inhibitory activity of normal human white blood cells in 38 cases averaged 37.4 per cent $\pm$ 8.7 per cent (S.D.) per 10^4 cells, and the inhibitory activity of normal marrow cells (seven cases) was 14.4 per cent $\pm$ 4.9 per cent per 10^4 cells (Table 1). The difference between these two tissues is, obviously, the presence of immature cells in the marrow and of mature cells of the same series in the blood. Hence, the difference in activity of the two types of cell preparations would seem to be due to the difference in cellular maturity between the two.

Lymphosarcoma and Hodgkin's disease.—In these two related diseases the inhibitor activity of the blood (nine cases) and the marrow (five cases) was not significantly different from the normal. Both the blood and marrow were, also, morphologically quite normal (Table 1). These data bear out the findings noted above and may, reasonably, be included with them. The smaller standard deviations are probably the result of improvement in technic as the work progressed.

Myelocytic leukemia.—In five cases of chronic myelocytic leukemia, under treatment, the mean inhibitor activity of the white blood cells was also quite close to normal (Table 2). Although the total white cell counts varied between 2,500 and 48,000, there were few immature cells present.

However, in two cases of leukemia, in relapse, the increase in the immaturity of the white blood cell pattern was paralleled by a considerable reduction in the inhibitor activity to 10.8 per cent. One of these patients (H.H.) was tested in both conditions, so that a direct comparison is possible (Table 2).

In contrast to the blood of treated leukemia, which showed a normal cell pattern and inhibitor content, the corresponding bone marrow exhibited a cell pattern of great immaturity and a correspondingly low inhibitor activity of 6.3 per cent. In leukemia, in relapse, the cellular composition of the blood approximated that of the bone marrow, and, similarly, the inhibitor concentrations were of the same order of magnitude.

In acute leukemia the considerably greater de-

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gree of cellular immaturity of both the blood and marrow was again paralleled by a further reduction in the amount of inhibitory activity. Here again, the cellular composition and the inhibitor concentration of both blood cells and marrow cells were of the same order of magnitude. Of interest is the blood inhibitor value in case E.C. which was close to normal when the cellular composition of the blood was not greatly disturbed.

It is evident, from these data, that it is not the leukemia, itself, but the state of maturity of the cells that determines the inhibitory activity of the cells. The same conclusion applies to lymphosarcoma and Hodgkin's disease.

Lymphocytic leukemia.—In this disease a striking contrast is seen between the inhibitory activity of blood and marrow cells in chronic lymphocytic leukemia and in acute lymphocytic leukemia (Table 3). In the chronic disease, the relatively high inhibitory activity is associated with an essentially lymphocytic cell pattern. In the acute leukemia, the absence of inhibitory activity is

TABLE 1

THE DESOXYRIBONUCLEASE INHIBITOR IN THE BLOOD AND BONE MARROW CELLS FROM THE NORMAL AND FROM LYMPHOSARCOMA AND HODGKIN'S DISEASE

PER CENT INHIBITION PER 10^4 CELLS		DIFFERENTIAL CELL COUNT						
Blood	Marrow	Polymorphs.	Metamyl.	Myel.	Pro-M.	Blast.	Lymph.	Normoblast
			Normal					
37.4±8.7		57					39	
	14.4±4.9	46	13	9	2			23
			Lymphosarcoma and Hodgkin's disease					
32.4±6.0		62					38	
	14.8±2.9	44	13	21	1	1		15

TABLE 2

THE DESOXYRIBONUCLEASE INHIBITOR IN MYELOCYTIC LEUKEMIA

PATIENT	PER CENT INHIBITION PER 10^4 CELLS		DIFFERENTIAL CELL COUNT						
	Blood	Marrow	Polymorphs.	Metamyl.	Myel.	Pro-M.	Blast.	Lymph.	Normoblasts
				Chronic leukemia (treated)					
S.E.	35.4		71					24	
H.H.	35.8		87					13	
M.F.	40.0		82					17	
H.W.	26.2		70(23)	10	7	4		9	
		2.6	46(22)	13	27	1			13
A.B.	22.1		62	5	3			29	
		9.9	51(23)	11	28	2			6
Mean	31.9		74	3	2	1		19	
		6.3	49	12	28	2			10
				Chronic leukemia (in relapse)					
L.B.	6.9		21(7)	20	30	24	5		
		7.4	17(7)	20	33	26	4		
H.H.	14.7		33(15)	13	33	18	3		
		7.2	22(5)	4	35	14	25		
Mean	10.8		28	17	32	21	4		
		7.3	20	12	34	20	15		
				Acute leukemia					
E.C.	34.1		78(9)	5	2	1	6	8	
		3.1	27	1			71		1
B.C.	2.0		33(7)	10	8	10	34	5	
		5.0	5(2)	12	33	40	10		
M.W.	6.4					98			
		0.0			16	40	44		
Mean	4.2*		16	5	4	71		3	
		2.7	11	4	13	27	42		

* E.C. omitted from this mean for obvious reason.

associated with the primitive lymphoblasts characteristically present. In both cases the similarity in cellular composition of the blood and the marrow is paralleled by a similarity in inhibitory activity. Here, again, it appears that the amount of inhibitor is related to the degree of cellular maturity.

It is of further interest that the blast cells of acute leukemia showed an excess of enzyme, even though large numbers of cells were used per test. Evidently, the inhibitor content is reduced to such an extent that the amount of desoxyribonuclease

TABLE 3

THE DESOXYRIBONUCLEASE INHIBITOR IN LYMPHOCYTIC LEUKEMIA

Patient	Per cent inhibition per 10^4 cells		Differential cell count						
	Blood	Marrow	Polymorphs.	Metamyl.	Myel.	Pro-M.	Blast.	Lymph.	Normoblast
Chronic lymphocytic leukemia									
A.S.		5.2	0					97	3
D.V.	6.4		10					89	
C.W.	6.5		2					98	
S.L.	18.2		19					81	
A.G.	13.5		8					92	
		12.0	6		1			89	4
W.D.	14.2		16					84	
		5.9	15(2)	3				75	7
P.G.	17.9		16					75	
		7.4	11					87	2
M.G.	10.8		15					85	
		11.6	31(8)	4	1	1		54	9
H.M.	13.1		5					94	
S.J.	7.9		4					94	
		2.6	6(3)	2	5			85	2
A.M.	12.1		10					88	
		12.1	2					95	2
	—	—	—	–	–	–		—	–
Mean	12.1		11					88	
		8.1	10	1	1			83	4
Acute lymphocytic leukemia									
								Blast. and lymph.	
M.W.	0*		0					100	
		0	0					100	
G.C.	0*		6					94	
E.B.	0*		4					96	

* Excess enzyme present.

normally present is demonstrable. The possible alternative, that an increase in enzyme had occurred without change in the amount of inhibitor, is less likely in light of the fact that Greenstein (6) has been unable to find changes in desoxyribonuclease in malignant tissues. In either case, the physiological or biochemical result would be the same.

DISCUSSION

The demonstration of an inhibitor of desoxyribonuclease casts light upon a possible mechanism regulating desoxyribonuclease activity (12, 13). The fact that this inhibitor, or similar ones, are present in a variety of cells, e.g., yeast (12, 13), various mammalian organ tissues, cancer (4, 5), and also in human white blood and marrow cells, both normal and malignant, would indicate that the inhibitor is of general biological significance. Zamenhof and Chargaff (12) theorize that the inhibitor is an important aspect of the system which regulates the functional activity of desoxyribonucleic acid by controlling the rate of its depolymerization. Of basic importance to this theory is the extent to which desoxyribonucleic acid is involved in cellular processes. It is generally believed that it plays a fundamental role in hereditary processes (1) and also, possibly, in the metabolic behavior of the cell (2, 11).

The present demonstration of a relationship between the concentration of the inhibitor and the degree of cellular maturity seems to suggest that the inhibitor has a significant role in the processes of cell maturation. Mature blood cells, which have ceased all growth and development, may be considered to have done so partly as a consequence of the cessation of desoxyribonucleic acid metabolism brought about by an inhibition of depolymerization. These cells are characterized by an absence of cytoplasmic ribonucleic acid and, it is believed, by a cessation of protein synthesis (11). On the

other hand, immature cells, in which cell multiplication (with a concomitant increase in the quantity of nucleoprotein and protein) and maturation are still active show lower concentrations of enzyme inhibitor, which would permit changes in desoxyribonucleic acid to occur at controlled rates of speed. Primitive blast cells, an important characteristic of which is a maximum rate of nucleoprotein production, contain little or no inhibitor, which would permit the above processes to operate at maximum speed. This theory, however, rests upon the demonstration that the degradation of desoxyribonucleic acid is a crucial factor in the above processes in which the rate of depolymerization is rate-determining.

SUMMARY

1. An inhibitor of desoxyribonuclease has been demonstrated in normal human white blood and bone marrow cells and in those from leukemia.

2. A direct relationship is shown to exist between the degree of cellular maturity of these cells and their inhibitory activity. The most mature white cells of the blood contain a maximum and the primitive blasts of leukemia contain a minimum of inhibitor. Cells of intermediate degrees of maturity contain intermediate amounts of inhibitor.

3. The above facts are discussed in light of a possible mechanism for the control of cell maturation and development.

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The Metabolism of 2-Aminofluorene in the Rat*

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The study of the metabolism of 2-aminofluorene (AF) has become of considerable importance, since it has been demonstrated that 2-acetylaminofluorene (AAF) is rapidly deacetylated in the rat (8, 13). This observation indicates that AF is a major product in the degradation of AAF by the rat and that the metabolism of AAF may be identical with that of AF. The metabolism of AF is also of interest from another point of view. AAF, which is readily hydrolyzed by the rat to AF, is a powerful carcinogenic agent. On the other hand, 2-benzoylaminofluorene[1] and 2-*p*-toluenesulfonamidofluorene, which are more resistant to hydrolysis *in vivo* (12), have proved to be either weakly carcinogenic or not carcinogenic.[2] These findings suggest that the free amino group may be required for the carcinogenic action of this class of amines.

The metabolism of AF has been previously investigated in the rabbit by Westfall (14). The quantity of acetone-extractable, diazotizable material in various tissues was estimated 18 hours following the subcutaneous administration of the compound. Only small amounts of nitrogen-conjugated compound were found after hydrolysis of the extracts with hydrochloric acid, demonstrating conjugation of the aromatic amino group. The data appeared to conform with subsequent results obtained for the metabolism of AAF by Westfall and Morris (15). These investigators showed that only 32 per cent of ingested AAF could be recovered 4 hours after administration if it was determined by diazotization and coupling with sodium 2-naphthol-3,6-disulfonate (R-salt) after previous hydrolysis with hydrochloric acid. It appeared that the compound had been altered during metabolism in such a manner as to be no longer diazotizable.

The present study extends the observations of Westfall (14), concerning the metabolism and distribution of AF in the rabbit, to the rat. The concentrations of diazotizable material were estimated from pooled samples following a single intraperitoneal injection. As in the rabbit, the amount of diazotizable material was increased by acid hydrolysis of the tissue extracts. There was a drop in the yield of diazotizable material similar to that observed with AAF.

To investigate the role of the liver in the metabolism of AF, and specifically with regard to the disappearance of the aromatic amino groups, rats were partially hepatectomized; following this procedure the distribution of the compound was studied.

Inasmuch as the level of hepatic riboflavin in the rat has been shown to influence the rate of destruction of a variety of carcinogenic compounds, such as *p*-dimethylaminoazobenzene and a number of related derivatives (6), it appeared that the metabolism of AF might be affected in a like manner. Consequently, measurements of the amount of diazotizable material after the intraperitoneal administration of AF were also made on the tissues of rats which had been deprived of riboflavin for 8–10 weeks.

MATERIALS AND METHODS

Animals.—The animals used in the experiments were male rats of the Sprague-Dawley strain weighing 200–300 gm.

Diet.—Prior to the administration of AF, the rats were maintained for 10–14 days on the following diet:

Casein, vitamin-free	180
Glucose	730
Cottonseed oil	50
Salt mixture (5)	40

The following vitamins were added to each 1,000 gm. of basal diet:

Choline chloride	0.3000
Calcium pantothenate	0.0070
Thiamine hydrochloride	0.0030
Pyridoxine hydrochloride	0.0025
Riboflavin	0.0200
Inositol	0.4000
Nicotinic acid	0.0200

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[1] Unpublished observations by J. H. Peters, H. R. Gutmann, and F. E. Ray.

[2] Personal communication of Dr. H. P. Morris to Dr. F. E. Ray.

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The rats in which the effect of riboflavin on the metabolism of AF was studied also received this diet, but without riboflavin, for 8–10 weeks. By then, these animals had lost about 11 per cent of their initial weight. These rats also showed progressive loss of hair, conjunctivitis, and increased irritability. All rats were allowed food and water ad libitum.

Partial hepatectomy.—Rats were partially hepatectomized by the removal of the median and left lateral lobes 3 days prior to the administration of AF. Following the operation the rats were returned to their cages and were allowed food and water freely.

Preparation of compounds and administration of AF.—AF, m.p. 127.5°–129.5° C.,[3] was synthesized from fluorene (7). 2-Amino-7-hydroxyfluorene, m.p. 265°–267°, was prepared from 2,7-diaminofluorene hydrochloride (3).

All rats received 100 mg. of AF/kg of body weight by intraperitoneal injection. Preliminary experiments had established that this dose was tolerated by the rat. For administration, the compound was dissolved in 2–3 ml. of warm propylene glycol. Following injection, the animals were placed in metabolism cages which permitted the separate collection of urine and feces. Water was allowed ad libitum, but food was withheld during the experimental period. The urine was collected in receivers to which a few drops of concentrated hydrochloric acid had been added and which were cooled by ice.

Preparation of tissues.—At the times stated in the tables, the animals were anesthetized with ether, and blood was obtained by heart puncture. In a few instances, the heart was exposed and the blood collected from an incision. Clotting was prevented by the addition of heparin to the collecting vessel. Equal weights of the heparinized blood were pooled and suitable aliquots prepared in duplicate. The samples were centrifuged at 2,000 r.p.m. for 30 minutes. The plasma was separated from the red cells and weighed. The red cells were next washed with 1 ml. of cold saline and the wash liquid added to the plasma. The diazotizable aromatic amino groups in the red cells and plasma were subsequently extracted and estimated as described below. The liver, kidneys, spleen, stomach, small intestine, caecum, and colon were removed, blotted on filter paper, weighed, and each tissue worked up separately. The heart, lungs, adrenals, thymus, pancreas, brain, diaphragm, esophagus, gonads, and bladder were combined in one fraction, designated as "miscellaneous," and the weight of this fraction was determined. The organs and the miscellaneous fraction were minced separately with scissors, and suitable aliquots were pooled. Duplicate samples, weighing in most instances 1 gm., were removed from the pool and homogenized with 3 ml. of distilled water by means of a Potter-Elvehjem homogenizer. The remaining carcass (skin, bones, muscles, and subcutaneous fat) was autoclaved at 15 lbs. pressure for 25 minutes. When subjected to the same procedure, AF was recovered in a 96 per cent yield. The autoclaved material was homogenized in a Waring Blendor with 0.1 M sodium hydroxide saturated with sodium chloride. The weight of the sodium hydroxide solution was twice that of the autoclaved carcass. The homogenates were combined and stirred mechanically for 15–20 minutes. Three-gm. aliquots containing 1 gm. of solid material were withdrawn from the pool for the extraction and subsequent measurement of diazotizable amino groups. The contents of the gastrointestinal tract (stomach, small intestine, caecum, and colon) were carefully removed and dried for 24 hours at 60° C. The dry solid was mixed with an equal weight of sand previously washed with dilute sulfuric acid and water and ground in a mortar to a fine powder. The aliquots of the ground material which were removed for extraction and subsequent analysis contained approximately 1 gm. of dry excreta.

Extraction and estimation of diazotizable aromatic amino groups.—The procedure for the extraction of the diazotizable material was a modification of that of Westfall (14). The samples were stirred mechanically for 10 minutes with 10 ml. of redistilled acetone and left at room temperature for 1 hour. They were centrifuged and the supernatant fluid decanted into 125-ml. Erlenmeyer flasks. The extraction of the residue was repeated, with stirring, 3 times with 10-ml. portions of 92 per cent acetone. The acetone extracts were combined, the flasks immersed in a water bath at 45°–50°, and the acetone removed by evaporation in a current of dry air.

The urine was pooled, filtered, and made up to a suitable volume, usually 50 ml. The pH was adjusted to 9 (hydrion paper) with concentrated ammonium hydroxide, and the solution extracted 3 times with an equal volume of diethyl ether. The ether was evaporated as described above.

Following evaporation of the solvent, the residues were transferred in two successive portions of 2 ml. of hot glacial acetic acid to 10-ml. volumetric flasks. The Erlenmeyer flasks were rinsed with hot distilled water and the wash liquid added to the volumetric flasks. The contents of the flasks were made up to 10 ml. with distilled water,

[3] All melting points are uncorrected.

mixed thoroughly, and cooled in an ice-salt bath. The mixtures were filtered through hard, fluted filters which retain any solid fat. The estimation of diazotizable amino groups was performed on aliquots of the filtrates by the following adaptation of the method of Westfall (14), which doubles the sensitivity and permits measurement of 1.0 μg. of AF in a total volume of 7.8 ml. Two-ml. aliquots were placed into each of two test tubes, and 0.3 ml. of concentrated hydrochloric acid and 0.5 ml. of distilled water were added. One ml. of distilled water was added to one of the tubes which served as the blank, while 1 ml. of 0.029 M sodium nitrite was delivered into the other tube. The mixtures were shaken and, after exactly 1 minute, poured into 3 ml. of 0.031 M sodium 2-naphthol-3,6-disulfonate in 10 M aqueous ammonium hydroxide. After the solutions had been cooled to room temperature in an ice bath, 1 ml. of acetone was added. The optical density of the resulting dye solution was determined immediately at a wave length of 525 mμ with a Beckman model DU spectrophotometer and expressed in μg. of AF by reference to a previously established calibration curve. The quantities thus obtained were designated as "free diazotizable material."

Two other aliquots of the filtrate were hydrolyzed prior to diazotization and coupling according to the method of Westfall and Morris (15). The amounts of diazotizable groups obtained following acid hydrolysis are due to "free diazotizable material" plus additional amino groups which were previously not available for diazotization. The aromatic amino groups present in the acid-hydrolyzed samples are referred to as "total diazotizable material." Appropriate corrections were made for the very small amounts of diazotizable compounds normally present in rat tissues. The above method gave recoveries of AF added to tissue homogenates which ranged from 80–100 per cent.

RESULTS AND DISCUSSION

The concentrations of free and total diazotizable compounds in the tissues of the rat 4 and 12 hours after the administration of AF are recorded in Table 1. It may be seen that aromatic amino groups were found in all tissues analyzed but that the concentration varied considerably. At 4 hours the concentration of total diazotizable material ranged from 21.0 μg/gm of tissue in the plasma to 431.0 μg/gm of tissue in the miscellaneous fraction; and at 12 hours from 20.5 μg/gm of tissue in the spleen to 73.2 μg/gm of tissue in the small intestine. Since AF is fat-soluble, it is possible that the lipid content of a tissue may be one of the factors which determines the early distribution of the compound. The high initial concentration of diazotizable material in the miscellaneous fraction is striking. This fraction contains several tissues with a high lipid content such as the brain, the adrenals, and the gonads, and it may be of interest to investigate the distribution of diazotizable material within the separate organs of this fraction.

It will be noted that all tissues contained free diazotizable material, but that additional amino groups became available for diazotization on heating with hydrochloric acid. It remains to be determined whether acetylation is involved in this reaction.

The recovery and distribution of the administered material are shown in Table 2. At 4 hours, 70 per cent of the administered amino groups were accounted for by hydrolysis, diazotization, and coupling with R-salt. About 10 per cent of these were present in the conjugated form. Fifty-seven per cent of the total diazotizable amino groups re-

TABLE 1

THE CONCENTRATION OF FREE AND TOTAL DIAZOTIZABLE MATERIAL IN RAT TISSUES 4 AND 12 HOURS AFTER A SINGLE INTRAPERITONEAL INJECTION OF 100 MG. OF 2-AF/KG OF BODY WEIGHT

	μG. OF DIAZOTIZABLE MATERIAL PER GRAM OF WET WEIGHT			
	(4 hours)*		(12 hours)*	
TISSUE	free	total	free	total
Carcass	55.3± 2.1	63.5±4.0	18.0±0.0	25.2±1.7
Miscellaneous	388.0±12.0	431.0±6.0	37.0±6.8	57.0±8.0
Liver	69.0± 4.0	73.5±1.5	51.7±0.2	60.5±1.5
Whole blood	24.7± 0.7	23.8±1.0	25.6±1.5	27.5±2.9
Plasma	19.0± 1.0	21.0±1.0	27.7±0.2	28.3±0.0
Red cells	30.4± 0.4	36.5±1.0	23.1±2.6	26.6±5.5
Small intestine	41.8± 2.2	56.0±1.5	18.7±0.6	73.2±2.3
Kidney†	42.8± 1.4	65.7±0.9	37.5±1.3	40.5±0.6
Stomach	14.8± 1.2	59.5±4.0	6.6±0.6	24.1±0.6
Caecum	138.0± 1.8	137.0±1.5	25.3±2.2	47.5±0.5
Colon	62.5± 4.5	72.0±7.0	22.8±2.8	32.0±4.0
Spleen	10.0± 1.1		14.8±0.8	20.5±1.5

* The analyses were performed on the pooled samples of six rats.

† The concentrations represent average values for the right and left kidney.

covered were found in the carcass and 31 per cent in the miscellaneous fraction, while liver and blood accounted for 3.5 and 2.8 per cent, respectively. In contrast to AAF, which does not penetrate the red cell (10), diazotizable material was present in the red cells and plasma in approximately equal amounts. The contents of the gastrointestinal tract contained 1.4 per cent of the recovered material, while only 0.1 per cent was excreted in the urine. The available evidence indicates that both the urinary and the gastrointestinal tracts are routes for the elimination of the compound.

Twelve hours after the administration of AF, 29.8 per cent of the injected amino groups were detectable by the diazotization technic, 9.2 per cent of these being conjugated. The decrease in diazotizable amino groups resembled qualitatively the disappearance of AAF observed by Westfall and Morris (15). Most of the tissues showed a drop in the recovery of injected diazotizable compound, the largest decrease having occurred in the carcass and the miscellaneous fraction. However, these tissues still accounted for 59 per cent of all amino groups present after 12 hours. The amount of diazotizable compounds obtained from the liver and blood remained practically unchanged. The maintenance of a high level of diazotizable material in the liver is of interest in view of the observation that this organ is one of the preferred sites of tumor induction with AF (8). The urine showed a 46.5-fold rise of diazotizable material, while the contents of the gastrointestinal tract increased only about 2.5-fold. It appears that more of the carcinogen is eliminated through the urinary tract than through the gastrointestinal route during the period from 4 to 12 hours.

Following partial hepatectomy 104.9 per cent of the injected aromatic amino groups was recovered after 4 hours, while 57.1 per cent was found 12 hours after the administration of AF. These values are to be contrasted to yields of 70.3 and 29.8 per cent, respectively, obtained for intact rats. A study of the distribution of diazotizable material 12 hours after the administration of the compound shows that most of the tissues of the hepatectomized animals contained 2–3 times as much of the injected amino groups as did the tissues of intact rats. The lower yield of diazotizable compound obtained from the liver following hepatectomy was undoubtedly the result of a decrease in the size of the liver. Although a rise in the concentration from 60.5 to 85.5 μg/gm of liver was observed, this did not compensate for the differences in tissue mass. The smaller amount of administered diazotizable material found in the small intestine of hepatectomized rats may indicate that the rate of disappearance of AF from this organ is accelerated when part of the liver has been removed. The data support the conclusion that the liver is one of the sites concerned with the metabolism of AF and also that the extent of disappearance of diazotizable material depends on the amount of active liver tissue. It will also be seen from Table 3 that conjugation of the amino group was observed after partial hepatectomy.

TABLE 2

DISTRIBUTION OF DIAZOTIZABLE MATERIAL IN RAT TISSUES AFTER A SINGLE INTRAPERITONEAL INJECTION OF 2-AF IN PER CENT OF THE ADMINISTERED DOSE*

Tissue	Per cent recovery of diazotizable material (4 hours)†		(12 hours)†		
	free	total	free	total	B/A
	a	A	b	B	
Carcass	33.86	40.34	10.59	14.83	0.37
Miscellaneous‡	20.22	22.46	1.88	2.88	0.13
Liver	2.33	2.48	1.97	2.30	0.93
Whole blood	1.66	1.94	1.72	1.85	0.95
Plasma	(0.83)	(0.97)	(0.98)	(1.00)	
Red cells	(0.83)	(0.97)	(0.74)	(0.85)	
Small intestine, contents	0.08	0.72	0.43	1.95	2.70
Small intestine	0.50	0.67	0.24	0.92	1.40
Kidney	0.29	0.45	0.29	0.36	0.80
Stomach, contents	0.10	0.38	0.15	0.87	2.33
Stomach	0.07	0.28	0.04	0.15	0.54
Caecum	0.23	0.23	0.05	0.09	0.39
Colon	0.18	0.21	0.06	0.08	0.38
Urine	0.02	0.07	3.09	3.25	46.50
Abdominal fluid	0.00	0.02	0.08	0.14	7.00
Caecum, contents	0.00	0.02	0.00	0.05	2.50
Spleen	0.02	0.01	0.02	0.03	
Total	59.56	70.28	20.60	29.75	

* 100 Mg. of 2-AF/kg of body weight was injected.

† Analyses were performed on the pooled samples of six rats.

‡ The miscellaneous sample consisted of brain, pancreas, diaphragm, bladder, gonads, esophagus, heart, thymus, lungs, and adrenals.

The depletion of riboflavin had no appreciable effect on the over-all yield of the administered diazotizable material, 56 and 35 per cent having been recovered 4 and 12 hours after the injection of AF. It would appear then that the level of tissue riboflavin is not related to the rate of disappearance of the injected aromatic amino groups. In this connection, it is of interest that the riboflavin content of the diet was previously found to be without effect on the incidence of tumors produced by AAF (4).

It is possible that the discrepancies noted in the distribution of diazotizable material between the riboflavin-depleted and the normal rat may not be the result of a lack of tissue riboflavin, but may be caused by changes in the weight and composition of the tissues incident to the riboflavin deficiency. Thus, the miscellaneous fraction of the normal rat which contained about 22 per cent of the injected diazotizable material weighed 85 gm. On the other hand, the miscellaneous fraction of the riboflavin-depleted rat weighed only 67 gm.

and accounted for about 9 per cent of the administered dose.

The chemical reaction by which the nitrogen of AF was rendered nondiazotizable in the experiments described above is not known. The condensation of AF with pyruvic acid (11) which would account for the loss of diazotizable amino groups has not been demonstrated *in vivo*. An alternative explanation for the disappearance of the diazotizable groups is suggested by certain properties of 2-amino-7-hydroxyfluorene. This compound (or its acetylated derivative) is to be regarded as a 2′-naphthol-3′,6′-disulfonate is only 37 per cent of that given by sodium fluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate. It follows that the oxidation of AF to 2-amino-7-hydroxyfluorene results in an apparent decrease of aromatic amino groups if they are measured by diazotization and coupling with R-salt and expressed in terms of AF. Likewise, the disappearance of the amino groups of AAF observed by Westfall and Morris (15) may be attributed to the oxidation of AAF to 7-hydroxy-2-acetylaminofluorene. Westfall and Morris (15) subjected their tissue extracts to hydrolysis

TABLE 3

DISTRIBUTION OF TOTAL DIAZOTIZABLE MATERIAL IN THE TISSUES OF THE PARTIALLY HEPATECTOMIZED OR RIBOFLAVIN-DEPLETED RAT AFTER A SINGLE INTRAPERITONEAL INJECTION OF 2-AF*

(As per cent of the administered dose)

	RECOVERY OF TOTAL DIAZOTIZABLE MATERIAL IN THE PARTIALLY HEPATECTOMIZED RAT		RECOVERY OF THE TOTAL DIAZOTIZABLE MATERIAL IN THE RIBOFLAVIN-DEPLETED RAT	
TISSUE	(4 hours)† per cent	(12 hours)‡ per cent	(4 hours)‡ per cent	(12 hours)§ per cent
Carcass	100.94#	39.52	37.20	23.09
Miscellaneous		6.62	8.76	1.64
Liver	1.71	1.74	3.69	2.18
Whole blood	1.62	3.05	4.08	3.19
Plasma		(1.56)	(1.77)	(1.72)
Red cells		(1.49)	(2.31)	(1.47)
Small intestine, contents		1.80	0.37	0.77
Small intestine		0.39	0.34	0.34
Kidney		0.74	0.75	0.59
Stomach contents			0.08	0.45
Stomach		0.31	0.13	0.22
Caecum		0.19	0.17	0.07
Colon		0.22	0.13	0.09
Urine	0.69	1.67	0.01	1.99
Abdominal fluid		0.50	0.36	0.26
Caecum, contents		0.25	0.01	0.05
Spleen		0.11	0.08	0.05
Total	(69.61)‖ 104.96	(46.28)‖ 57.11	(44.75)‖ 56.16	(22.60)‖ 35.03

* 100 Mg. of 2-AF/kg of body weight was injected.

† Analyses were performed on the pooled samples of two rats.

‡ Analyses we re performed on the pooled samples of six rats.

§ Analyses were performed on the pooled samples of five rats.

\# This value includes all tissues listed except liver, blood, and urine.

‖ These values represent the per cent recoveries calculated in terms of free diazotizable material.

likely product of the *in vivo* oxidation of AF. Since the isolation of 7-hydroxy-2-acetylaminofluorene from rat urine (1), it has been recognized that the 7-position of the fluorene molecule is subject to metabolic attack.

2-Amino-7-hydroxyfluorene can be diazotized and coupled with R-salt to form sodium 7-hydroxyfluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate which absorbs maximally at a wave length of 530 mμ (3). AF gives sodium fluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate with an absorption maximum at 525–530 mμ (15). 2-Aminofluorene cannot be distinguished therefore from 2-amino-7-hydroxyfluorene by diazotization and coupling with R-salt. It will be noted from Chart 1 that the extinction due to sodium 7-hydroxyfluorenyl-2-azo- with hydrochloric acid prior to diazotization and coupling. Since it has been reported by Goulden and Kon (2) that 2-amino-7-hydroxyfluorene is destroyed on boiling, tests of the stability of 2-amino-7-hydroxyfluorene were carried out with the same concentration of hydrochloric acid, temperature, and duration of heating as was used by Westfall and Morris (15). The data of Table 4 show that the compound is stable under these conditions. Consequently, 2-amino-7-hydroxyfluorene may have been present in the hydrolysates prepared by Westfall and Morris (15) and may have been responsible for the apparent decrease of diazotizable material.

It is concluded that the disappearance of diazotizable amino groups may be due to the presence

of the hydroxylated derivatives of AF or AAF in the tissues of the rat, both in the present experiments and in those reported by Westfall and Morris (15). Further experiments involving the isolation of the oxidation products of AF and AAF are in progress.

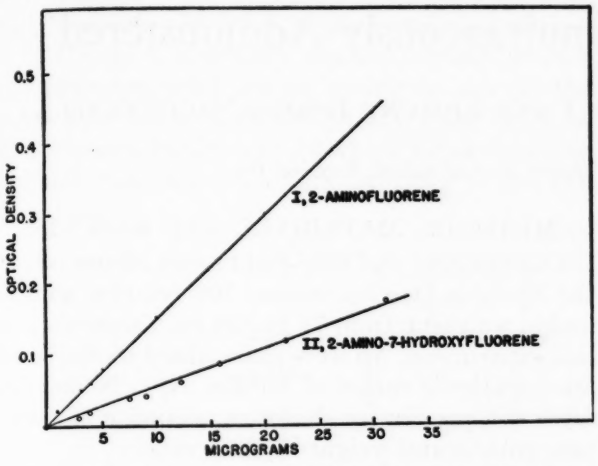

CHART 1.—The relationship between the weight of 2-aminofluorene and 2-amino-7-hydroxyfluorene and the optical densities of sodium fluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate (I) and sodium 7-hydroxyfluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate (II).

TABLE 4

THE STABILITY OF 2-AMINO-7-HYDROXYFLUORENE TO HEATING AT 100° FOR 1 HOUR WITH HYDROCHLORIC ACID

Compound	Micrograms present before heating	Micrograms present after heating	Per cent recovery
2-Amino-7-hydroxy-fluorene*	23.8	23.5	
	23.8	23.8	
	av. (23.8)	av. (23.7)	99.4

* The test system consisted of 2-amino-7-hydroxyfluorene in 0.5 ml. of glacial acetic acid, 0.3 ml. of concentrated hydrochloric acid, and 2.0 ml. of distilled water.

SUMMARY

1. The distribution of diazotizable material following a single intraperitoneal injection of AF has been studied in the rat. The aromatic amino group was present in both the free and the conjugated form. A drop in the yield of diazotizable material with time, similar to that previously described for AAF, was observed.

2. The effects of partial hepatectomy and depletion of riboflavin on the metabolism of AF were investigated. The higher recovery of diazotizable compound in the partially hepatectomized rat indicates that the liver is concerned with the disappearance of the aromatic amino groups. No appreciable effect of the depletion of riboflavin on the metabolism of AF was noted.

3. The optical densities of sodium fluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate and sodium 7-hydroxyfluorenyl-2-azo-2′-naphthol-3′,6′-disulfonate have been compared, and the stability of 2-amino-7-hydroxyfluorene has been determined. On the basis of the data, it is suggested that the apparent disappearance of the aromatic amino groups of AF and AAF is due to *in vivo* hydroxylation of the compounds.

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Liver Tumor Inhibition and Adrenal Histologic Responses in Rats to Which 3′-Methyl-4-dimethylaminoazobenzene and 20-Methylcholanthrene Were Simultaneously Administered*

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This study was undertaken to investigate by histologic methods the cellular changes in the liver and other organs in rats treated with two different carcinogens. Lacassagne (3) and recently Riegel and associates (6) have reported that, when 1,2,5,6-dibenzofluorene is administered, there is a reduced tumor incidence resulting from methylcholanthrene. Richardson and Cunningham (5) demonstrated independently that the application of methylcholanthrene resulted in a lowering of liver tumor incidence in rats fed diets containing 3′-methyl-4-dimethylaminoazobenzene (m′-Me-DAB). Jaffé (2) fed 4-dimethylaminoazobenzene and simultaneously gave a single intraperitoneal administration of 20-methylcholanthrene (MCA) and concluded that MCA did not affect the action of the azo carcinogen under the experimental conditions employed.

In this experiment, six groups of rats were studied concurrently. These rats were given the carcinogens 3′-methyl-4-dimethylaminoazobenzene (m′-Me-DAB) and 20-methylcholanthrene (MCA), either alone or simultaneously. The animals and groups were studied comparatively for the following conditions: (*a*) survival rates; (*b*) time required for carcinogenesis; (*c*) the anticarcinogenic effect with MCA administered vaginally, subcutaneously, orally, and peritoneally; *d*) types of tumors produced; (*e*) the frequency and distribution of metastases; (*f*) the effects of each carcinogen separately or in combination on the adrenal cortex and other organs.

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METHODS, MATERIALS, AND RESULTS

One hundred and fifty-eight adult albino rats of the Sprague-Dawley strain, 103 females and 55 males, weighing from 75 to 350 gm., were used in this experiment. All were maintained on the basal semi-synthetic ration of Griffin, Nye, Noda, and Luck (1), previously shown as adequate to maintain growth and weight in these rats.

Group 1 consisted of 54 rats (29 males and 25 females). These were given the basal diet, to which 0.06 per cent m′-Me-DAB was added. The animals lived 15–29 weeks. They died from tumor complications, pneumonia, or were sacrificed because of their moribund condition.

Group 2, sixteen female rats, was fed the basal synthetic diet, and 0.1 ml. of an 0.6 per cent solution of MCA in benzene was instilled weekly into the vagina. These animals died or were sacrificed because of a moribund condition from the first to the 55th week.

Group 3, made up of 24 female rats, received the basal diet, to which 0.06 per cent m′-Me-DAB had been added. They also received weekly vaginal instillations of 0.1 ml. of 0.6 per cent MCA in benzene. They died or were sacrificed over a period of 7–35 weeks.

Group 4 included twenty rats, fifteen females and five males, and received the basal diet to which was added 0.06 per cent m′-Me-DAB and 0.0067 per cent MCA by weight. These animals survived from 2 to 34 weeks, at which time the remaining three animals were sacrificed.

Group 5, comprised of twenty rats, eleven females and nine males, received the basal diet, to which 0.06 per cent m′-Me-DAB was added, and 0.126 gm. of crystalline MCA was implanted under the skin of the back of each animal. This was done through a dorsal mid-line incision which was closed with interrupted catgut sutures. In fifteen animals nembutal anesthesia was used and in five ether anesthesia. This group lived from 10 to 40 weeks.

Group 6, twelve female and twelve male rats, was fed the basal diet plus 0.06 per cent m′-Me-DAB and had 0.126 gm. of crystalline MCA placed into the peritoneal cavity through an anterior mid-line incision. The muscles were closed with continuous and the skin with interrupted catgut sutures. Nembutal anesthesia was used for eight animals and ether in the remaining sixteen.

In Group 1, mortality was the highest between the 19th and 26th weeks, owing to the m′-Me-DAB liver-induced cancers. Only three survived 27–29 weeks. In Group 2, in which MCA alone was instilled vaginally, the survival rates of the rats were not materially affected. The longest survival was 55 weeks. In Groups 3, 4, and 5, the longest survival rates were 35, 34, and 40 weeks, respectively. Of Group 6, in which crystalline MCA was placed into the peritoneal cavity, 42 per cent died within 2 weeks, owing to chemical peritonitis. The longest survival was 27 weeks (Chart 1).

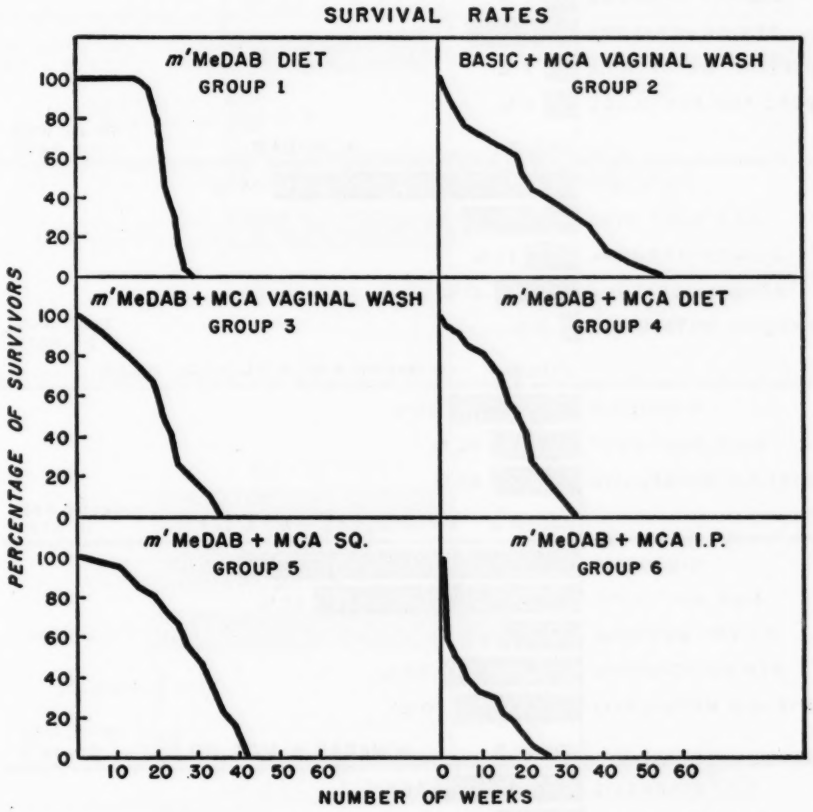

CHART 1.—Survival rates

All animals were autopsied. All organs, including the hypophysis, thyroid, adrenals, gonads, lymph nodes, bone marrow, and the visceral organs, were examined histologically. The tissues were fixed in either Vandegrift's solution (7) or 10 per cent formaldehyde and were stained with hematoxylin-eosin. All lobes of the liver and lungs were sectioned, and the adrenal glands received particular attention.

The histologic findings in the 54 animals of Group 1 (fed 0.06 per cent m′-Me-DAB in the basal diet) were the subject of a previous report (4). Ninety-eight per cent of these rats developed liver cancer in a period of 15–29 weeks. This group serves as a control in the portion of this study dealing with the liver tumor-inhibitory effect of simultaneously administered carcinogens.

The benign liver changes found in 54 rats (of Group 1) living 15–29 weeks were: nodular cirrhosis, 53 (98 per cent); biliary duct cysts, 29 (54 per cent); biliary adenomas, four (7 per cent); benign hepatomas, fifteen (28 per cent); liver myeloid metaplasia, four (7 per cent); and metaplastic change represented by bone and cartilage formation in four (7 per cent) (Chart 2).

Malignant liver tumor types found were: malignant hepatomas, small- and large-cell types, 51 (94 per cent); anaplastic carcinoma, seven (13 per cent); adenocarcinoma, liver-cell type, 30 (55 per cent); adenocarcinoma, bile duct type, 38 (70 per cent); and liver sarcoma, five (9 per cent) (Chart 3). Metastases occurred in 50 (94 per cent): to the lungs, 40 (75 per cent); lymph nodes, 25 (47 per cent); peritoneal cavity, 47 (89 per cent); adrenal,

one (2 per cent); and heart, three (6 per cent) (Chart 4). All modes of metastases—implantation, direct extension, and venous, arterial, and lymphatic spread—and all types of liver cancers were represented.

The adrenal glands showed no significant change (Figs. 1 and 2).

The sixteen female rats of Group 2 which were maintained on a semi-synthetic diet and received

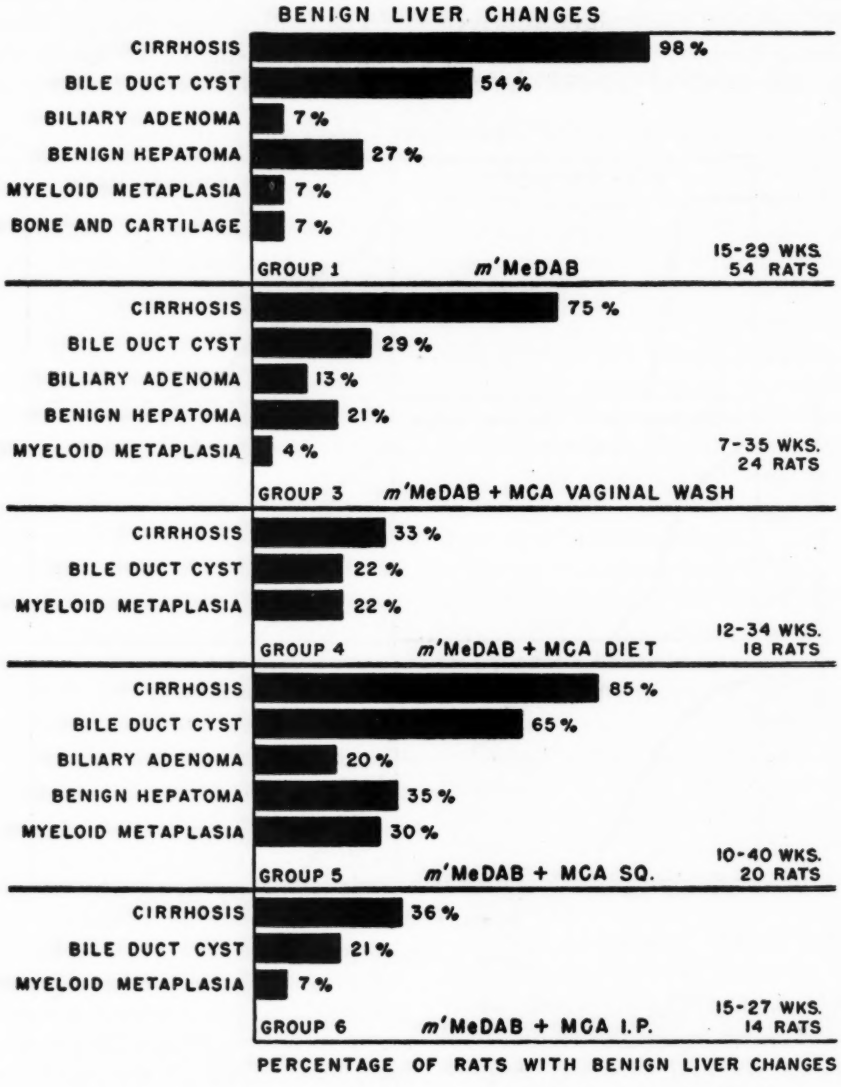

CHART 2.—Incidence of benign liver changes in various treatments

sisting of 24 rats, liver biopsies were performed every 14 days. The earliest liver neoplasm was observed at 28 weeks. The following benign liver changes in 24 rats living 7–35 weeks were: nodular cirrhosis, eighteen (75 per cent); biliary duct cysts, seven (29 per cent); biliary adenomas, three (13 per cent); benign hepatomas, five (21 per cent); and liver myeloid metaplasia, one (4 per cent) (Chart 2).

MCA vaginally each week did not differ essentially from the animal colony controls. Vaginal neoplastic changes did not occur. Hypertrophy and hyperplasia of the adrenal cortex was found in the two rats which were on the carcinogenic regime 55 weeks, but in no other organs were there significant changes.

In Group 3 (intravaginal MCA weekly and 0.06 per cent m'-Me-DAB in the basal diet), con-

Malignant liver tumors were first observed at 28 weeks and developed in five rats (21 per cent). In all five the tumors were adenocarcinomas of liver-cell types; in two there were also malignant hepatomas. Neoplasms which could be identified as bile duct in origin were not found in this group. All the malignant liver tumors were liver-cell in type (Chart 3). Metastases occurred in one case (20 per cent) and was confined to local extension

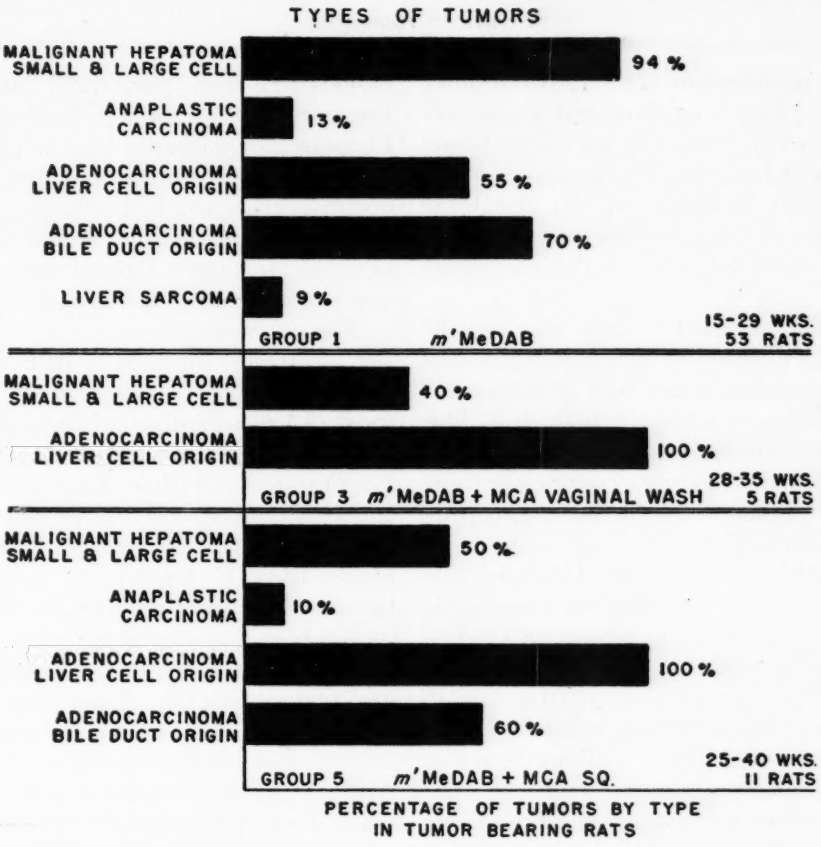

CHART 3.—Incidence of malignant liver tumors in various treatments. (Note: In Groups 2, 4, and 6, there were no malignant liver tumors.)

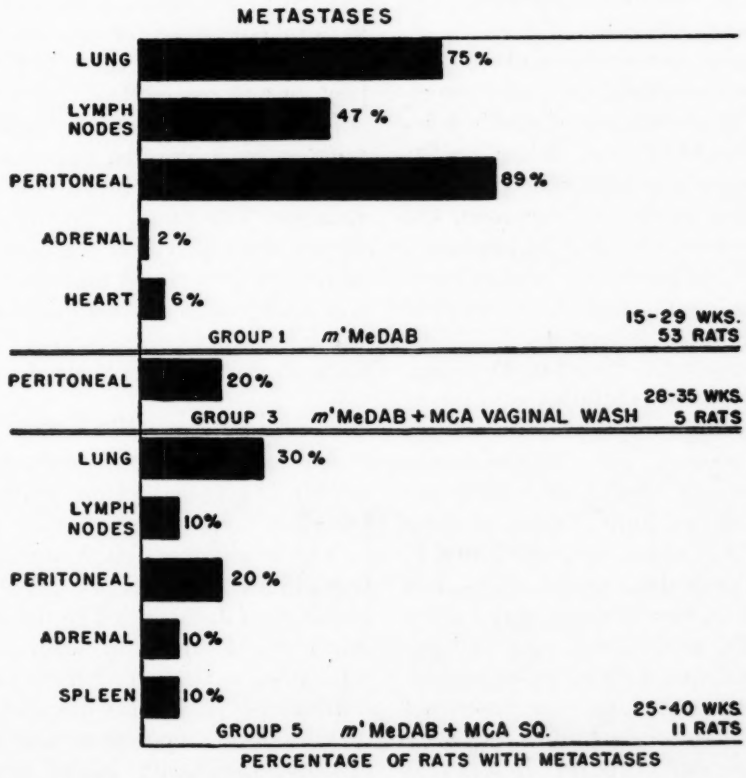

CHART 4.—Frequency and sites of metastases of live tumors produced by various treatments. (Note: In Group 5, subcutaneous fibrosarcomas were produced. Group 6 also had fibrosarcomas but no malignant liver neoplasms.)

into the adjacent peritoneum. The most common neoplasm in this group was liver-cell adenocarcinoma of small-cell type, and it was this tumor that metastasized (Chart 4). The two malignant hepatomas remained localized within the liver parenchyma. None of the lesions were grossly visible and were found only on histologic sectioning.

After the vaginal application there was a rapid loss of the carcinogen, since by ultraviolet fluorescence the methylcholanthrene had disappeared from the vaginal mucosa after the fifth day. The estrous cycle was undisturbed, as shown by the vaginal smear. However, in spite of poor retention, the anticarcinogenic effect was sufficient to produce liver tumor inhibition.

In Group 4 (MCA and m′-Me-DAB in the basal diet), used to study the effect of simultaneous oral administration of two carcinogens, the benign liver changes in eighteen rats living 12–34 weeks were: minimal cirrhosis, six (33 per cent); bile duct cysts, four (22 per cent); myeloid metaplasia, four (22 per cent) (Chart 2). Tumors in the liver or other organs did not occur.

The most significant, consistent, and outstanding histologic changes were in the cortex of the adrenal gland. These were as follows: hypertrophy and hyperplasia, six (30 per cent); fatty metaplasia and lipoid storage, twelve (60 per cent); and atrophy, six (30 per cent) (Fig. 3).

Group 5 (MCA subcutaneously and m′-Me-DAB in the basal diet) was used to study the effect of subcutaneous implantation of methylcholanthrene when m′-Me-DAB was taken orally. The benign liver changes in twenty rats living 10–40 weeks were: nodular cirrhosis, seventeen (85 per cent); biliary duct cysts, thirteen (65 per cent); biliary adenomas, four (20 per cent); benign hepatomas, seven (35 per cent); and liver myeloid metaplasia, six (30 per cent) (Chart 2).

The first liver neoplasm was found in 25 weeks. Malignant liver tumors were as follows: malignant hepatomas, small- and large-cell types, five (50 per cent); anaplastic carcinoma, one (10 per cent); adenocarcinoma, liver-cell origin, ten (100 per cent); and adenocarcinoma, bile duct origin, six (60 per cent) (Chart 3). Metastases were found in the lungs in three (30 per cent); lymph nodes, one (10 per cent); peritoneal, two (20 per cent); adrenal, one (10 per cent); and spleen, one (10 per cent) (Chart 4). Metastases were all of adenocarcinoma of the liver-cell type. One case presented gross metastasis, and in three the metastases were in the form of microscopic thrombi. In one rat metastases were microscopically present in the adrenal, spleen, and periaortic lymph nodes. In two cases there were peritoneal implants, confirmed microscopically. An MCA-induced subcutaneous fibrosarcoma occurred in four (20 per cent).

The adrenals contained the following benign cortical changes: hypertrophy and hyperplasia, eight (40 per cent); atrophy, five (25 per cent) (Fig. 4); and fatty metaplasia and lipoid storage, one (5 per cent) (Figs. 5 and 6).

The experiments on Group 6 (m′-Me-DAB in the basal diet and MCA intraperitoneally) were the most difficult to interpret and evaluate, since most of the animals died early, and since methylcholanthrene, when placed in the peritoneal cavity, produces multiple granulomatous lesions. Of the 24 rats used, ten (42 per cent) died within the first 2 weeks. The remaining fourteen all lived longer than 15 weeks, but none developed liver tumors. The liver changes in these fourteen rats living 15–27 weeks were: cirrhosis, five (36 per cent); biliary duct cysts, three (21 per cent); myeloid metaplasia, one (7 per cent) (Chart 2). Four survived 19, 23, 24, and 26 weeks, respectively, but showed no liver tumors. However, sarcomas with diffuse local extension did occur in these animals. Three (21 per cent) were fibrosarcomas, and one (7 per cent) was a mixed angio- and fibrosarcoma.

In the adrenals the following histologic changes, limited to the cortex, were found: hypertrophy and hyperplasia, nine (38 per cent); fatty metaplasia and lipoid storage, three (13 per cent); and atrophy, one (4 per cent).

The lungs, kidneys, hypophysis, thyroid, thymus, salivary glands, muscles, arteries, intestinal viscera, and the brain showed no significant changes. The ovaries and testes were in various stages of atrophy. This change was not confined to any single group and was not to be correlated with any carcinogen or combination of carcinogens; similar changes were present in the control animals on the semi-synthetic basic diet.

DISCUSSION

An anticarcinogenic effect of MCA against m′-Me-DAB production of liver tumor in rats was found in these experiments.

The amount of MCA simultaneously administered in our experiments was 60 times greater than in those of Jaffé (2). The decrease in cancer incidence when MCA is administered concurrently with m′-Me-DAB, either by vaginal wash or subcutaneously, is mainly due to the marked decrease of malignant hepatomas and bile duct adenocarcinomas ordinarily found when m′-Me-DAB is given alone. When cancer developed, adenocarcinoma of liver-cell origin was the predominant

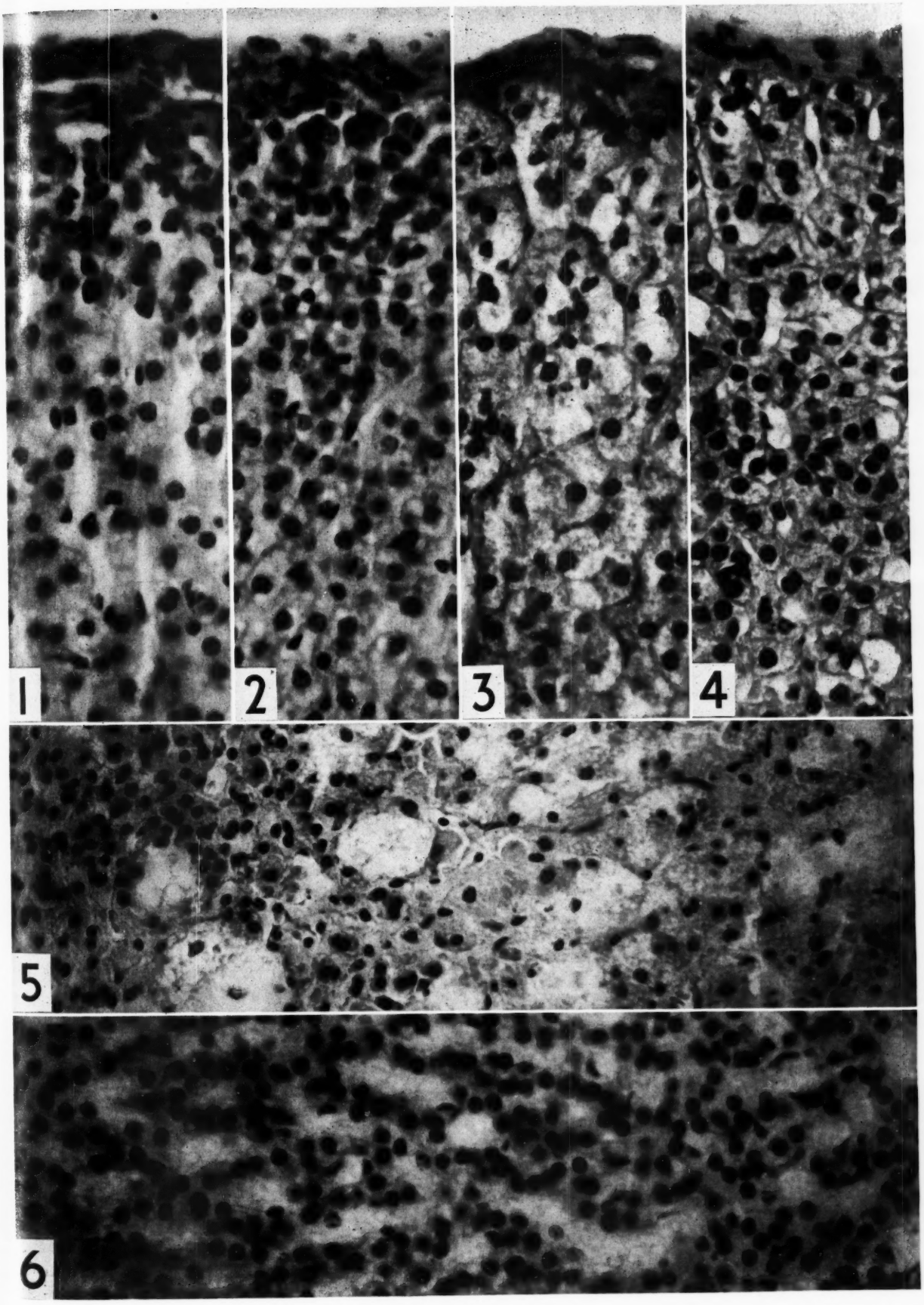
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FIG. 1.—Rat on control basal diet 20 weeks to show the normal adrenal zona fasciculata and glomerulosa. ×460.

FIG. 2.—Rat on m′-Me-DAB (Group 1) 26 weeks to show the adrenal zona fasciculata and glomerulosa. ×460.

FIG. 3.—Rat on m′-Me-DAB and MCA in the diet (Group 4) 34 weeks, to show the adrenal atrophy of the zona fasciculata and glomerulosa. ×460.

FIG. 4.—Rat on m′-Me-DAB and MCA subcutaneously (Group 5) 40 weeks, to show the adrenal atrophy of the zona fasciculata and glomerulosa. ×460.

FIG. 5.—Rat on m′-Me-DAB and MCA subcutaneously (Group 5) 40 weeks, to show adrenal fatty metaplasia and lipoid storage in the zona reticularis and fasciculata. ×400.

FIG. 6.—Rat on control basal diet 10 weeks to show a normal adrenal zona reticularis and fasciculata for comparison. ×400.

neoplasm (as it is from the administration of m′-Me-DAB alone).

In our experience, every case of m′-Me-DAB-induced hepatoma was preceded by marked cirrhosis. In the rats in which MCA was administered simultaneously with the m′-Me-DAB, the incidence of cirrhosis was decreased in proportion to the decrease in frequency of malignant hepatomas.

Neoplasia of both hepatic cells and bile duct epithelium was inhibited in these experiments, with the greatest inhibitory influence shown against bile duct neoplasms.

When the liver carcinogen and MCA were simultaneously administered, no stromal metaplastic changes, such as cartilage or bone formation, were seen such as were found in the rats receiving the liver carcinogen alone.

Liver tumor inhibition may also indicate that there is absorption and concentration of the MCA. Absorption varies in the different anatomic sites. When MCA was administered orally and intraperitoneally, absorption was good and resulted in complete inhibition of liver tumor induction by the m′-Me-DAB. In contrast, MCA absorption was poor when intravaginally and subcutaneously administered, as indicated by the partial inhibition of liver tumor formation. The MCA, when subcutaneously located, rapidly forms a fibromatous granuloma and, when placed in the vaginal tract, precipitates on the vaginal epithelium and hence is lost during the estrous desquamation.

In Groups 1 and 2, in which m′-Me-DAB and MCA were each separately administered, the adrenal cortical change could not be attributed to the carcinogen. In Groups 3, 4, 5, and 6, the animals simultaneously received MCA and m′-Me-DAB. Only in Group 3 did the adrenal show no cortical histologic change. The animals receiving the carcinogens orally showed the greatest incidence of adrenal cortical atrophy.

These adrenal cortical changes are the subject of further investigations now in progress.

SUMMARY AND CONCLUSIONS

1. Five Sprague-Dawley strain rat groups were fed ad libitum a basal semi-synthetic diet containing 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene (m′-Me-DAB). Four of these groups were simultaneously treated with 20-methylcholanthrene (MCA) by the following methods: dietary, peritoneal cavity implantation, subcutaneous implantation, and vaginal lavage.

2. Rats on m′-Me-DAB lived no longer than 29 weeks, dying of tumor complications. Life was prolonged up to 40 weeks in rats receiving m′-Me-DAB and the polycyclic carcinogen simultaneously.

3. Liver tumor development was either delayed or entirely inhibited by the simultaneous administration of the carcinogens m′-Me-DAB and MCA. Bile duct tumors were the type most inhibited.

4. Cirrhosis of the liver was either delayed or entirely inhibited by the simultaneous administration of the carcinogens m′-Me-DAB and MCA.

5. Metastases decreased in incidence. Adenocarcinoma of the liver-cell type was the only type involved in a local extension or confined to microscopic thrombi.

6. The combined administration of MCA and m′-Me-DAB produced morphologic adrenal cortical change.

7. In spite of liver tumor inhibition, lack of cirrhosis, and the adrenal cortical morphologic changes, MCA fibrosarcomas occurred in the animals receiving MCA subcutaneously and intraperitoneally.

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The Influence of Certain Variables on the Incidence of Gastric Neoplasia in Mice of the Br-S Strain*

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INTRODUCTION

The origin of the Br-S strain was described in 1945 when it had reached the fifteenth generation of brother-sister inbreeding (1). The strain was characterized by chocolate color[1] and a high incidence of gastric neoplasia near the pylorus.

At that time the Br-S strain was divided into two sub-lines: the first to continue as before, with routine subcutaneous injection of all the mice at 60 days of age with 0.1 cc. sesame oil containing 1 mg. methylcholanthrene; and the second sub-line to continue by inbreeding but without methylcholanthrene (untreated descent). A study of this untreated sub-line has been reported (4), showing that gastric tumors continued to appear with high frequency. Incidence at successive ages was found to rise to a plateau by about 250 days, except in the case of breeding females, whose gastric tumors reached a plateau when mice were 1 year and were consistently low in incidence.

A report on the treated sub-line was presented in 1947, with evidence that the gastric tumors were being developed at progressively earlier ages with advancing generations (2). It is the purpose of the present paper to analyze further developments in this treated line, with a consideration of certain variables which may influence the incidence of gastric neoplasia in mice.

MATERIALS AND METHODS

Except in special tests which will be explained below, it has been the practice to keep all the mice of this stock as breeders, usually three or four to a box. Their care has remained as uniform as possible; the details are described in earlier papers (1). The basic food has been Nurishmix (Pratt Food Co.), with weekly supplements of bread, milk, cod-liver oil, and mixed grains (wheat, oats, and calf-meal pellets). Lettuce was formerly given also but has been omitted in the present experiment.

In the course of the breeding, several color changes (mutations, probably) and occasional mammary tumors appeared in one branch of this strain, and a special effort was made to multiply this sub-strain, referred to as "3 CAMG." Data on this group will be treated separately below.

Observations

Since May, 1945, approximately 2,500 mice of the Br-S treated line have been injected with methylcholanthrene and completed their life-span. This interval covers about eleven generations (F_{13}–F_{24}).

The importance of litter seriation in a related strain (3) as a factor in response to methylcholanthrene prompted a similar analysis here. However, no apparent relation exists between litter seriation and incidence of either local or gastric tumors in mice of the Br-S strain (Chart 1). Subdivision of the data on the basis of sex shows a sex difference of considerable importance in incidence, but which is unrelated to litter seriation.

A most significant revelation appeared when the data were broken into successive time-interval groups, according to date of birth. As shown in Chart 2, there were striking fluctuations of incidence of both types of tumors; the sex difference is quite apparent, male and female curves paralleling one another up and down, but not crossing. The most surprising feature is the dropping out of the gastric tumors, the incidence in the last period being nearly zero.

A similar chronological analysis of the data from the 3 CAMG sub-line, separated from the main Br-S line long before the decline, demonstrated that a similar drop in gastric tumor incidence had occurred there also. Moreover, even the untreated line showed the same phenomenon.

The evidence would seem clear enough that the decline in gastric tumor incidence had nothing to

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[1] Chocolate differs from standard wild mouse color by two recessive genes, *aa* and *bb*.

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do with the methylcholanthrene treatment, but further analysis is possible. Multiplying the incidence of local tumors by the incidence of stomach tumors, we get a product which would theoretically be the simultaneous incidence of both types of tumors if they were independent in origin and cause. For the total data, the figures are 73.4 per cent (local) × 26.4 per cent (gastric) = 19.4 per cent ± 0.8 per cent. This theoretical value is almost exactly what was observed, namely, 19.6 per cent. Similarly, breaking the data down according to successive time intervals and calculating as before, we get very good agreement in most cases.

The above agreement between calculated and observed incidences might be thought to settle the question, but one puzzling fact remains. The mice developing local tumors show a distribution of latent periods which is unimodal, with medians fluctuating between 145 and 180 days. This means that a large proportion—up to one-half—of the mice with local tumors were too young to have their full incidence of gastric tumors. An analysis of the ages of the gastric tumor cases (with and without local) shows a definitely *bimodal* distribution. The early mode seems strongly influenced by the presence of local tumors. Thus, to put the matter in different terms, the mice with local tumors at younger ages have a greater share of gastric tumors than would be expected, while those at later ages have a smaller share. By adding all together we obtain an average which agrees with calculations.

A Special Test

A separate experiment was set up to determine the possible relation of gastric tumor incidence to methylcholanthrene injection. Mice of the 3 CAMG sub-line were used; they were of the 20th to 24th generations of inbreeding and were born in intervals F and G (Chart 2). Their ancestors had all been treated with methylcholanthrene. The 3 CAMG mice differ from the typical Br-S in having a white frontal spot ("blaze") and a white belly streak; the ventral pelage is pale.

Three groups were set up for this test, each group consisting of about 100 mice. One group received methylcholanthrene injections as usual; the second was not injected and was scheduled to be killed at the same age as the first; the third group also was not injected, but was not to be killed until a year old. The mice were kept in groups of about five to the box, with the sexes separate. Only Nurishmix pellets were given as food, with no supplements. Otherwise they had the customary care of this laboratory.

When mice of the first group developed definite local tumors they were killed, weighed, and autopsied. Tumor sizes were recorded by a sketch, and representative tumors were preserved for histological study. Some mice died without local tumors; provided they had not decomposed, they were autopsied as usual, but body weights were omitted. Mice which developed no local tumors by

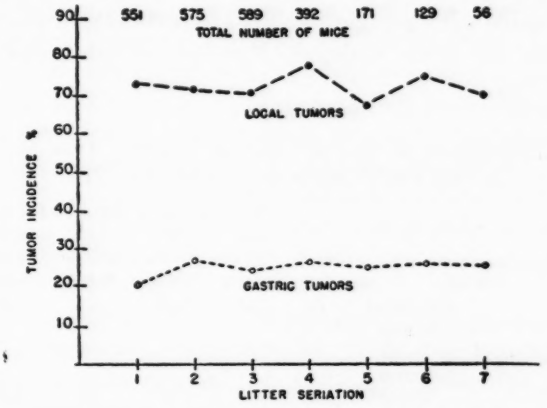

CHART 1.—Incidence of "local" tumors and of gastric tumors in Br-S mice according to the order of the litters in which they were born.

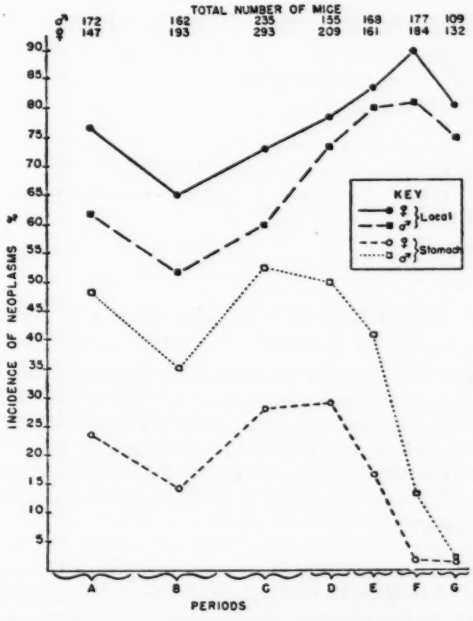

CHART 2.—Incidence of "local" tumors and of gastric tumors in Br-S mice born in successive time intervals. Beginning May, 1945, intervals A, B, and C are about a year each, while D, E, F, and G are about half a year each. The last interval extends into early 1950.

the age of a year were then killed. In the second group, the mice were killed so as to be similar to those of the first group with regard to age and sex. A few died at various ages.

The data on age and tumor incidence are presented in Table 1. The differences between groups

(*a*) and (*b*) are apparently negligible, and the increased incidence of gastric tumors in group (*c*) is of little significance, because of the difference in median ages.

An analysis of body weight distributions (Chart 3) reveals a clear difference with regard to presence or absence of gastric tumors. The median weights for mice with gastric tumors are several gm. above those for mice without. Further analysis shows

TABLE 1

INCIDENCE OF "LOCAL" AND OF GASTRIC TUMORS IN "3 CAMG" MICE OF THE SPECIAL TEST

Group		Number	Median Age at Autopsy	Per cent with local tumor	Per cent with gastric tumor	Per cent with both tumors
Injected	with local tumor	55	225	100	55	55
	no local tumor	48	340		50	
	Total	103	260	53	52	29
b) Controls		96	298		51	
c) Controls (older)		85	370		68	

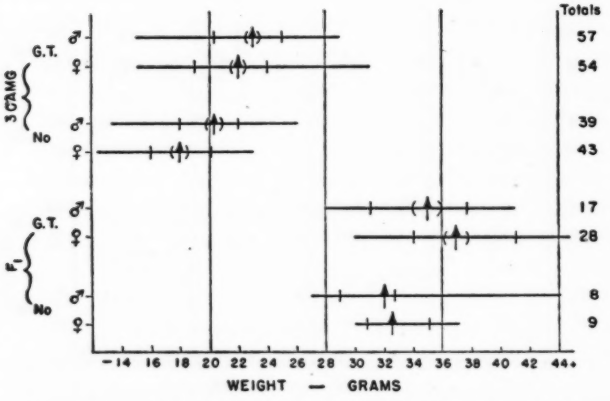

CHART 3.—Analysis of frequency distributions of body weights at autopsy for mice of the "3 CAMG" sub-line in the special test and for F_1 hybrids from 3 CAMG × C57 black. G.T. = gastric tumor found; No = no gastric tumor found. Ranges of distribution are shown by lines, medians by arrows, quartiles by cross bars, and ± probable errors of medians by parentheses.

that this is true in both sexes and is independent of methylcholanthrene treatment, local tumors, and age at autopsy. Furthermore, a positive correlation (about + 0.3) is found between size of the gastric tumor and body weight.

OBSERVATIONS ON AN OUTCROSS

During the last time interval (*G*, Chart 2), a group of F_1 hybrids from a cross of 3 CAMG × C57 black mice (nontumor) was raised without methylcholanthrene treatment. Most of these mice were allowed to breed freely; they were given only Nurishmix pellets (no supplements) and were killed at the age of 330–370 days; the males were killed earlier so that the females would not be pregnant at autopsy.

Several interesting facts are obtained from this group. First, in spite of heavy breeding, the females here show no reduction in incidence of gastric tumors as compared to the males—in fact, if any change, there is a rise. Second, the incidence is even higher in both groups than in the pure 3 CAMG stock, and in the Br-S. Third, this high incidence occurred at the same period of time that the incidence among mice of the Br-S strain was at nearly zero. Fourth, the body weight distributions for the hybrids (Chart 3) show a correlation with gastric tumors like that found in the 3 CAMG stock, in both sexes, though the body weight medians for the hybrids are much greater than those of the pure strains. Another point which is of interest is that a maternal influence does not appear in the crosses—gastric tumors appeared in the F_1 regardless of strain of mother.

DISCUSSION

This study has altered several previous opinions about the origin of gastric tumors in mice of the Br-S strain. Four principal variables have been considered: litter seriation, methylcholanthrene, successive time intervals, and body weight.

In view of the definite influence of litter seriation on incidence and latent period for methylcholanthrene-induced local tumors in some other strains (3), it is a surprise to find that it has no significance in strain Br-S. This is true not only for local tumors but also for gastric tumors. As a corollary of this finding, the absence of maternal influence in reciprocal crosses between Br-S and C57 black mice is to be noted.

Methylcholanthrene-treated mice on the whole have had an incidence of gastric tumors similar to that of untreated mice of this strain. Also, the total coincidence of induced local tumors and gastric tumors agrees very well with calculations. These facts, however, are only part of the story. In treated populations there is a differential effect at early and late ages: coincidence of both types of tumors at first is higher than calculated, later is lower. The following working hypothesis is proposed to explain the paradox: An unknown variable is responsible for differences among the mice in sensitivity to methylcholanthrene, as shown in latent periods and incidence of failure to develop a local tumor, even with a normal life-span. Very few of the mice develop local tumors beyond a year after injection. Presumably, the carcinogen is dissipated within a few months after injection, and mice developing stomach tumors late would tend

not to have local tumors. On the other hand, during early ages the same unknown variable affecting sensitivity to methylcholanthrene may similarly affect gastric tumor incidence. Thus, a high coincidence early would be balanced by a low value later, so that the over-all correlation would seem zero.

In methylcholanthrene-treated groups the age distribution for gastric tumors has tended to be bimodal, one peak close to that for local tumors, and the other much later. A bimodal distribution gives the average or median opportunity to shift wildly with relatively minor influences. In previous papers (1, 4) progressive reduction in average age occurring over several generations was noted and interpreted as genetic in nature. That interpretation is no longer exclusively tenable.

Nutritional factors are emphatically indicated as significant variables for gastric tumor incidence according to our remaining evidence. The marked drop in incidence in certain time intervals for the groups receiving dietary supplements, while incidences remained high for groups without supplements, points to an inhibitory effect of some ingredient in the supplements. However, the postulated inhibitory principle seems specific for gastric tumors, since local tumors during this period were not reduced in incidence.

The present data clearly show a relation between gastric tumors and body weight. Mice with the tumors tend to weigh 10–15 per cent more than comparable mice without tumors. Sex, presence of local tumors, and hybridity apparently do not disturb this correlation. Although careful study has yet to be made, it is our impression that much if not all the weight differential depends on body fat. Here again a nutritional factor seems implicated.

We may cite two different lines of nutritional research which have been successful in identifying factors of importance in carcinogenesis. One is commonly referred to as "caloric restriction" (5); this condition has been found generally to inhibit tumor development. The other line of research concerns vitamins. Vitamin A seems particularly important in relation to methylcholanthrene sensitivity (6). Whether either or both of these types of nutritional factors are involved in the control of gastric tumor development we cannot know at present. However, the identification of such a factor, capable of completely inhibiting formation of gastric neoplasms in this strain of mice, should now not be too difficult.

SUMMARY

Analysis of data accumulated since 1945 for the Br-S strain has been made. Litter seriation (at least through the seventh litter) shows no influence on incidence of gastric tumors or of methylcholanthrene-induced local tumors in this strain. Incidences of gastric tumors and of local tumors are practically independent, over-all coincidence values agreeing with calculations. However, the age at incidence of gastric tumors tends to be bimodal, with positive correlation of gastric and local tumor incidence in the earlier age group, negative in the later.

Marked fluctuations of gastric tumor incidences with time are demonstrated in Br-S mice. A striking drop at one period was limited to those mice receiving dietary supplements. Two other groups at this time fed only Nurishmix had as high an incidence as usual. One of these groups consisted of F_1 hybrids from the gastric tumor strain × C57 black.

Body weights show a significant correlation with gastric tumor incidence. Mice with such tumors have median body weights about 10–15 per cent greater than those lacking them.

The evidence favors an interpretation of variables in nutritional status, not yet analyzed, which are capable of completely suppressing gastric tumors in this strain.

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Effect of Nitrogen Mustard on Tumor Incidence and Lethal Mutation Rate in Drosophila*

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One of the most stimulating findings in recent years is that of Auerbach and co-workers (2) that the nitrogen mustards are mutagenic. It has focused attention on the possibilities of chemical induction of mutations and has prompted numerous investigations. Nitrogen mustard has also been found carcinogenic (4, 11, 12, 13) for mice, as well as palliative in certain types of cancer in man. Therefore, in any study of the relation between mutations and tumors, the action of this chemical might well be examined carefully. In this investigation methylbis(2-chloroethyl)amine hydrochloride has been administered to a tumor strain of Drosophila, and the lethal mutation rate and tumor incidence have been determined simultaneously.

METHODS

Fifty males and 50 females from siblings of the *tu 36a* strain with a normal sex ratio were mated in a bottle containing 50 ml. of culture medium covered with growing yeast. When 3 days old these flies were treated with 1 per cent methylbis(2-chloroethyl)amine hydrochloride in propylene glycol, administered as an aerosol. The aerosol was generated intermittently for 30 seconds every 30 minutes for periods of both 24 and 48 hours. A control culture was prepared at the same time each experimental culture was treated and followed subsequently in the same manner. One transfer to a fresh bottle was made at the end of 3 days from the beginning of treatment. Separate records were kept for these cultures. Offspring from the treated flies were examined after eclosion, and the number of tumors was tabulated. A representative number of females from this generation were mated individually to scS^1 *B InS* w^a sc^8 males and the lethal mutation rate determined by the Muller-5 method. A more extended discussion of the method may be found in a previous publication (6).

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RESULTS

Ten per cent of the males from the group treated 24 hours bore tumors, but only 1.39 per cent of those without treatment were tumor-bearing (Table 1). The females in the treated group also had a higher percentage of tumors (5.66 per cent) than those in the control group (0.21 per cent). There were 15 tumor-bearing flies among 196 with antecedent treatment and only 7 among 904 without. All these differences in incidence are significant ($P < 0.0001$).

TABLE 1

EFFECT OF NITROGEN MUSTARD (24-HOUR ADMINISTRATION) ON TUMOR INCIDENCE

	Tumors	Population	Per cent tumorous	P
♂♂ Treated	9	90	10.00	<0.0001
♂♂ Without treatment	6	432	1.39	
♀♀ Treated	6	106	5.66	<0.0001
♀♀ Without treatment	1	472	0.21	
Total treated	15	196	7.65	<0.0001
Total without treatment	7	904	0.77	

TABLE 2

EFFECT OF NITROGEN MUSTARD (48-HOUR ADMINISTRATION) ON TUMOR INCIDENCE

	Tumors	Population	Per cent tumorous	P
♂♂ Treated	27	384	7.03	<0.0001
♂♂ Without treatment	3	362	0.83	
♀♀ Treated	19	392	4.85	<0.0001
♀♀ Without treatment	1	407	0.25	
Total treated	46	776	5.93	<0.0001
Total without treatment	4	769	0.52	

The results of 48-hour treatment on tumor incidence are given in Table 2. There were 27 males

and 19 females with tumors after treatment, in populations of 384 and 392, respectively—an incidence of 7.03 per cent in males and 4.85 per cent in females. In comparison, the control cultures contained only 3 tumors in 362 males and 1 tumor in 407 females. The incidence of 46 tumorous flies among 776 flies following treatment is significantly higher than 4 among 769 ($P < 0.0001$). Although they are not truly comparable in a temporal sense, the data for original and transfer cultures are presented in Table 3.

The lethal mutation rate (Table 4) was 2.04 per cent when treatment extended over a period of 24 hours, a significantly higher rate than the 0.09 per cent among those without treatment ($P < 0.0001$). In Table 5 the lethal mutation rate is given for the 48-hour treatment period with nitrogen mustard. There were 29 lethals among 781 chromosomes tested, an incidence of 3.71 per cent in the treated group, and only one lethal among 983 chromosomes tested in the control group, an incidence of 0.10 per cent. This difference in mutation rate is clearly significant ($P < 0.0001$).

DISCUSSION

The incidence of tumors is definitely higher after administration of nitrogen mustard to this strain of Drosophila. This is true when individual cultures are compared to parallel controls, for males, for females, and for the entire group considered as a whole. The fact that 48-hour administration results in no higher incidence than 24-hour treatment is probably most easily explained by the rapid disappearance of the active form of the chemical. Although it is tempting to ascribe the higher incidence of tumors in the transfer cultures to a delayed effect, the absence of significant differences statistically does not justify it.

Boyland and Horning (4) treated stock mice with weekly subcutaneous injections of methylbis-(2-chloroethyl)amine hydrochloride and methyltris(2-chloroethyl)amine hydrochloride. They found ten tumor-bearing mice out of fourteen surviving for more than 280 days. There were lung carcinomas and adenomas in eight, lymphosarcomas in two, one fibromyoma of the uterus, and one spindle-celled sarcoma at the site of injection. In a control group of 40 mice, killed between 14 and 18 months of age, six had adenomas of the lung and two had hepatomas. The tumors in treated mice were larger and had a more malignant appearance, and the earliest tumor was found at 284 days. Heston has shown that the incidence and average number of pulmonary adenomas in the A strain are increased by the intravenous injection of methylbis(2-chloroethyl)amine hydrochloride (12) and also by the same treatment with sulfur mustard, bis(2-chloroethyl)sulfide (13). Griffin, Brandt, and Tatum (11) report that Swiss mice and albino rats, treated intravenously, subcutaneously, and intraperitoneally with the same chemicals used by Boyland and Horning, developed tumors including adenocarcinoma of the lung, leukemia, lymphosarcoma, angioma, angiosarcoma, and osteogenic sarcoma. Any similarity between the origin of cellular growths in Drosophila and mammalian tumors is conjectural, but it is interesting that the incidence of certain examples of both types of tumors is increased by nitrogen mustard.

The mutation rate following treatment is much higher than the natural mutation frequency of this

TABLE 3

COMPARISON OF TUMOR INCIDENCE IN ORIGINAL AND TRANSFER CULTURES

	Culture	Tumors	Population	Per cent tumorous	P
♂♂ Treated	Original	16	277	5.78	0.078
	Transfer	20	197	10.15	
♀♀ Treated	Original	13	336	3.87	0.040
	Transfer	12	162	7.41	
Total treated	Original	29	613	4.73	0.008
	Transfer	32	359	8.91	
♂♂ Without treatment	Original	6	569	1.05	0.940
	Transfer	3	225	1.33	
♀♀ Without treatment	Original	0	592	0.00	0.043
	Transfer	2	287	0.70	
Total without treatment	Original	6	1,161	0.52	0.300
	Transfer	5	512	0.98	

TABLE 4

EFFECT OF NITROGEN MUSTARD (24-HOUR ADMINISTRATION) ON LETHAL MUTATION RATE

	Lethals	Chromosomes tested	Per cent lethals	P
Treated	11	540	2.04	<0.0001
Control	1	1,102	0.09	

TABLE 5

EFFECT OF NITROGEN MUSTARD (48-HOUR ADMINISTRATION) ON LETHAL MUTATION RATE

	Lethals	Chromosomes tested	Per cent lethals	P
Treated	29	781	3.71	<0.0001
Control	1	983	0.10	

strain for both 24- and 48-hour periods. This confirms the findings of Auerbach and Robson (2) and others, using tumor-resistant stocks. Nitrogen mustard is apparently a powerful mutagen in the hands of all who have used it, and conflicting reports (such as those made on the mutagenic effects of the carcinogenic hydrocarbons) are not encountered when this chemical is used.

The data presented indicate that both tumor incidence and mutation rate are increased simultaneously when nitrogen mustard is administered to a strain of tumor-bearing flies. The question of causal relationship between the increased mutation rate and increased numbers of tumors deserves some consideration. Before an affirmative answer is given too hastily, the results in other studies of similar design should be reviewed. It was found that 20-methylcholanthrene is tumorigenic but not mutagenic for this strain (9). Formaldehyde increased the mutation frequency in males but did not alter the incidence of tumors for either males or females (7). Diethylstilbestrol changed neither mutation rate nor tumor incidence (8). It is apparent that increased mutation rate following treatment is not always associated with increased numbers of tumors, and increased numbers of tumors after treatment are not always associated with higher mutation rate. The possibility that the combination of mutagenic and tumorigenic properties is fortuitous in the case of nitrogen mustard cannot therefore be eliminated at this time. The discrepancies mentioned and the lack of evidence for mutational effects of carcinogenic hydrocarbons in Drosophila (1, 3, 5, 9, 10) should be resolved before the evidence presented in this communication can be used to support the somatic mutation hypothesis of tumor etiology. The facts seem clear and are harmonious with the results of others, but the interpretation is best deferred in view of other current information. The double action of nitrogen mustard makes this group of chemicals and others with similar properties important tools in continued investigation of the causes of cancer.

CONCLUSIONS

1. Methylbis(2-chloroethyl)amine hydrochloride, administered as an aerosol to the *tu 36a* tumor strain of Drosophila, increased the tumor incidence significantly.

2. This chemical also caused a simultaneous increase in lethal mutation rate.

3. A causal relationship between these two properties of this chemical should not be assumed as necessarily true until certain discrepancies are resolved.

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The Effect of Folic Acid on A-Methopterin–induced Inhibition of Nucleic Acid Synthesis

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It has been observed that folic acid (2) or citrovorum factor (1) will prevent the anti-leukemic action of folic acid antagonists. We have reported that desoxyribonucleic acid (5) and vitamin B_{12} (7) will partially prevent the anti-leukemic action of 4-aminopteroylglutamic acids, and have more recently observed that a combination of thymidine, adenylic, guanylic, and cytidylic acids or DNA plus RNA plus B_{12} are more effective in preventing the anti-leukemic action of 4-amino-N^{10}-methylpteroylglutamic acid (A-methopterin) than any of these materials administered alone.[1]

It is known that folic acid antagonists will inhibit incorporation of formate into visceral nucleic acids of mice and rats (8, 3). In view of the fact that the anti-cancer activity of A-methopterin appears to be directed toward inhibition of nucleic acid synthesis, it was considered of interest to determine whether this inhibition could be prevented by folic acid.

EXPERIMENTAL

Experimental procedures employed herein have already been described in detail (8). Adult CFW mice treated with A-methopterin and with folic acid plus A-methopterin (in immediate succession) were injected with C^{14}-formate (1.0 μc.), and at 6 hours the viscera were excised, pooled, and the combined nucleic acids and then nucleic acid purines were isolated (8) and assayed for radioactivity (6). The dosage schedules and experimental results obtained are summarized in Table 1.

DISCUSSION

It seems quite evident from the data presented that folic acid at high levels (60 mg/kg) will partially prevent the inhibition of formate incorporation into mouse nucleic acids brought about by A-methopterin. Formate carbon also is known to be incorporated into the alpha carbon of serine and into glycogen (4), into the methyl group of methionine, and into the methyl carbons of choline (9). It can be seen from the data on visceral homogenates in Table 1 that A-methopterin has not significantly inhibited formate incorporation into tissue as a whole but has rather profoundly affected the incorporation of this precursor of the 2- and 8-carbon atoms of purines into nucleic acids (isolated from aliquots of the visceral homogenate). This would seem to suggest that, under the conditions of the present experiments, there is some degree of specificity in this inhibition.

TABLE 1

THE EFFECT OF FOLIC ACID ON A-METHOPTERIN-INDUCED INHIBITION OF NUCLEIC ACID SYNTHESIS

Exp. no.	Treatment	Dosage (mg/kg)	Specific activities (μc/mol carbon) Visceral homogenate	Combined nucleic acids	Nucleic acid purines
1	None		4.5	18.9	75.6
2	A-methopterin	3 (2×)	4.7	5.2	14.4
3	A-methopterin	2 (2×)	4.8	6.8	19.4
4	A-methopterin	1 (2×)	4.0	5.4	14.3
5	A-methopterin+folic acid	2+60 (2×)	4.6	10.3	37.2
6	A-methopterin+folic acid	1+60 (2×)		18.4	39.0

Note: Four mice were used in each experiment. About 1.0 μc. of formate was injected per mouse. The A-methopterin was administered intraperitoneally on the 2 days immediately prior to injection of C^{14}-formate. Folic acid was injected I.P., as indicated, immediately prior to the injections of A-methopterin.

[1] H. E. Skipper, M. Bell, and J. B. Chapman, unpublished data.

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SUMMARY

Using C^{14}-formate incorporation as a means of measuring the rate of synthesis of nucleic acids, it has been demonstrated that the inhibition of nucleic acid synthesis induced by A-methopterin can be partially reversed by folic acid.

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The Failure of C^{14}-Formate To Affect the Course of Mouse Leukemia*

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It was pointed out in 1948 by Dougherty and Lawrence (2) that "for neoplastic diseases in general, localizing substances of adequate selectivity that can be labeled and used for radiotherapy have not been discovered. If such are found, it would be desirable that they carry a radioactive isotope which emits a very soft ray, such as $_1H^3$ or $_6C^{14}$. Even these emit beta-rays that traverse several cell diameters; however, the half penetration of the $_1H^3$ beta-ray is less than the radius of the average cell."

The incorporation of formate carbon into the 2- and 8-positions of the uric acid skeleton was first observed by Sonne, Buchanan, and Delluva (4). Totter *et al.* (5) have shown that, likewise in the rat, formate is a precursor of desoxyribonucleic acid guanine, adenine, and thymine (methyl carbon) and ribonucleic acid guanine and adenine. We have confirmed these latter observations many times in the mouse.[1]

More recently, with the use of the blood smear autoradiogram technic, it has been found that leukemic cells (Ak_4 strain in Akm mice) are much more avid in the incorporation of C^{14} from formate than normal leukocytes (3). At 1 hour after the injection of 100 microcuries (μc.) of C^{14}-formate, 90 per cent of the leukemic cells were highly radioactive, as compared to about 5 per cent of the more mature lymphoid elements. No radioactive polymorphonuclear leukocytes, thrombocytes, or erythrocytes were observed at 1 hour. These observations suggested the possibility that high levels of C^{14}-formate might provide a degree of anti-leukemic action. The knowledge that formate is incorporated into chromosome components, a suspected site of the neoplastic mutation, as well as the short range of C^{14} beta particles, was considered encouraging. Knowledge of the low energy of carbon-14 disintegration (0.15 million electron volts) made the possibility less attractive.

EXPERIMENTAL

Two types of experiments have thus far been carried out to determine the possible chemotherapeutic activity of C^{14}-formate in transplantable leukemia. High levels of isotopic formate have been injected into Akm mice at 2 days after inoculation with Ak_4 leukemia and the life span of the treated mice compared to that of untreated leukemic controls. This general procedure for screening candidate anti-leukemic agents has been previously described (1). In a second and perhaps more sensitive assay of the effect of C^{14}-formate on leukemic cell formation, mice with advanced leukemia have been injected with isotopic formate and the action on hematopoiesis followed by total white blood cell counts.

TABLE 1

OBSERVATIONS ON THE EFFECT OF RADIOACTIVE FORMATE ON THE LIFE SPAN OF MICE WITH AK_4 LEUKEMIA

Treatment	Dosage (μc.)	Days of leukemic death* 6	7	8	9	10	11	12	Av. life span (days)
Experiment I:									
Inactive formate				4	2	3	1		9.1
Radioactive formate	200				3	1	1		9.6
Experiment II:									
Inactive formate				2	2		1		9.0
Radioactive formate	100			3	1	1			8.6

* Values are the number of mice dying on a given day.

The levels of isotopic carbon injected have been of the order of 100–200 μc. per mouse (approximately a 280–560 mc. man-equivalent). The results obtained regarding the effect of C^{14}-formate on the life span of mice with Ak_4 leukemia are presented in Table 1. The effect of 100 μc. of this material on the leukocyte counts of leukemic mice may be seen in results summarized in Table 2.

In still another type of experiment, the effect of high levels of C^{14}-formate on the pattern of deaths from spontaneous leukemia in Akm mice has been

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[1] L. L. Bennett, Jr., H. E. Skipper, J. Meade, M. Bryant, and L. Simpson, unpublished data.

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studied. This strain of mice, which is highly inbred, develops spontaneous leukemia in almost 100 per cent of cases at from 9 to 12 months of age. Paired litter-mates were divided into two groups of fifteen mice each. The ages of the animals at initiation of this experiment are indicated in Table 3. C^{14}-labeled sodium formate was injected intraperitoneally into one group at a level of 50 μc/month for 5 months for a total activity of about 250 μc/mouse. The control mice received injections of inactive sodium formate. The age of leukemic death of each animal in both groups is indicated in Table 3.

TABLE 2

THE EFFECT OF C^{14}-FORMATE ON THE WHITE BLOOD CELL COUNT OF MICE WITH AK_4 LEUKEMIA

	Total white blood cell count	
	Range	Av.
Controls (4 mice):		
5th day after transplant	7,800– 10,300	8,760
6th day after transplant	15,000– 26,800	22,000
7th day after transplant	52,600– 95,400	76,000
8th day after transplant	154,000–172,800	164,200
C^{14}-formate injected (4 mice):		
6th day after transplant*	17,800– 46,000	32,000
7th day after transplant	54,000–124,000	83,000
8th day after transplant	208,000–348,000	278,000†

* The mice of the experimental group received injections of 100 μc. of C^{14}-formate on the 6th day after leukemic inoculation.

† Only two mice alive on the 8th day.

Note: All blood counts were on tail vein blood.

TABLE 3

THE EFFECTS OF HIGH LEVELS OF C^{14}-FORMATE ON THE PATTERN OF DEATHS FROM SPONTANEOUS LEUKEMIA IN AKM MICE

Controls				C^{14}-Formate			
Litter	Sex	Age when injected (days)	Age at leukemic death (days)	Litter	Sex	Age when injected (days)	Age at leukemic death (days)
a	F	68	219	a	F	68	405
b	F	72	258	b	F	72	342
c	F	70	271	a	F	68	241
c	F	70	275	c	F	70	249
d	F	80	332	d	F	80	250
a	M	68	320	a	M	68	547
b	M	72	246	b	M	72	339
a	M	68	251	c	M	70	*
d	M	80	345	d	M	80	*
d	M	80	384	d	M	80	*
d	F	80	361	d	M	80	326
d	F	80	225	e	M	70	*
e	F	70	333	e	M	70	322
e	F	70	392	e	M	70	*
f	F	70	278	f	M	70	406
			Av. 299				Av. 343

* Nonleukemic deaths associated with fighting.

Note: The average life span of 107 untreated Akm strain mice dying of spontaneous leukemia in this laboratory was 280 days (σ51).

DISCUSSION

The data presented in Tables 1 and 2 indicate that C^{14} from sodium formate, when injected into mice with Ak_4 leukemia at the level of 100–200 μc/animal, has no perceptible effect on the leukemic process. The C^{14}-injected leukemic mice lived approximately the same period as nonradioactive formate-injected leukemic controls. Under the conditions of these experiments, C^{14}-formate had no tendency to inhibit leukemic cell proliferation (Table 2). This latter experiment is considered a rather sensitive assay of the radiation effects of C^{14}.

Although a disappointment from the standpoint of cancer chemotherapy, these results suggest that carbon-14 in fairly high doses (at least as formate) is not apt to have much effect on mammalian hematopoiesis.

It seems apparent that, under the conditions of present experiments, high levels of C^{14}-formate (a 700-mc. man-equivalent), given over a period of 5 months, have had no significant effect on the development of spontaneous leukemia in Akm mice.

There still exists the possibility that C^{11}-formate might be used for anti-leukemic activity. The more energetic beta radiation (0.99 Mev) and the short half-life (20.5 minutes) of carbon-11 might prove advantageous, although rapid synthesis of formate and utilization of a cyclotron for C^{11} production introduce technical complications.

SUMMARY

C^{14}-formate, when injected at levels of 100–200 μc/mouse (280–560 mc. man-equivalent), had no perceptible effect on life span or hematopoiesis of mice with Ak_4 leukemia. Approximately 250 μc/mouse, given over a 5 months' period, had no significant effect on the development of spontaneous leukemia in Akm mice.

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An Ultracentrifugal Analysis of the Macromolecular Particles of Normal and Leukemic Mouse Spleen

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As part of a comprehensive program of isolation and comparison of the various nucleoprotein fractions of normal and leukemic mouse spleen (10, 11), the macromolecular particles of the cytoplasm have been separated by differential centrifugation and studied in the analytical ultracentrifuge. Although these preparations contain large amounts of polydisperse material, there are also several well defined sedimentation boundaries. While the components which they represent are found only in small amounts in preparations made from normal spleen, their concentrations are markedly elevated in preparations from either transplanted or spontaneous leukemic spleen.

MATERIALS AND METHODS

Most of the experiments were carried out on mice of the Ak strain (2), with the same line of leukemia, 9421, as that used in previous studies (10, 11). When leukemic spleen, minced in saline, was injected intraperitoneally into normal mice 3 months old, the animals developed advanced leukemia in about 9 days. At this time they were sacrificed by spinal fracture, and the spleens were removed and chilled immediately. The spontaneous leukemic spleens were obtained from Ak mice 5–8 months old and, in one case, from a single C58 mouse. The normal spleens were obtained from healthy mice 3 months old. One normal control experiment was also made on Carworth Farms mice, in which leukemia is extremely rare.

The chilled spleens were weighed quickly and homogenized in 9 volumes of 0.88 M sucrose, as previously described (10), except for one experiment in which 0.1 M phosphate buffer, pH 8.0, containing 0.03 per cent heparin (7) was used.

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The procedure for the separation of the particles in sucrose is outlined in Chart 1. The entire procedure was carried out in the cold. The sedimentations at 20,000 × gravity were done in a Servall SS-1 centrifuge in the cold room, and those at 170,000 × *g* in a Spinco Model E ultracentrifuge.[1] The rotor was pre-cooled to 3° C. and filled in the cold room, and the vacuum chamber was refrigerated throughout the run. After high speed centrifugation the particles were resuspended in 2.5 cc. of cold buffer or sucrose per gram of spleen,

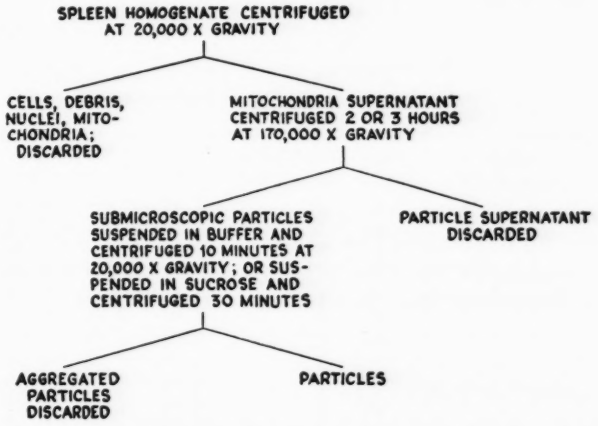

CHART 1.—The separation of cytoplasmic particles from mouse spleen homogenized in 0.88 M sucrose.

with the help of an electrically driven glass homogenizer pestle ground to fit closely in the plastic centrifuge tubes. Suspension of the particles in the buffer (0.06 M veronal containing 0.05 M sodium chloride, pH 8.5 and ionic strength 0.10) was achieved by first distributing them in a measured amount of distilled water and then adding an equal amount of double strength buffer. Aggregated particles were removed by centrifugation at 20,000 × *g* and the clear solution kept cold until just before it was put into the ultracentrifuge cell.

Sedimentation analyses were made in a 12-mm. cell, in the Spinco ultracentrifuge. Particles sus-

[1] Spinco Model E ultracentrifuge, Specialized Instruments Corporation, Belmont, Calif.

pended in buffer were run at 200,000 × *g*, with photographs at 2-minute intervals. For sucrose suspensions, a force of 250,000 × *g* was used, and photographs were taken at 4-minute intervals. All analyses were made at room temperature except for one experiment which was carried out in the cold.

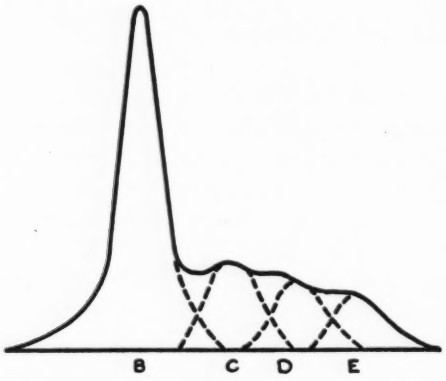

CHART 2.—The analysis of the sedimentation curve from Exp. 9a (spontaneous leukemia, in buffer).

The sedimentation constants were corrected to 20° C. for the effect of temperature on the viscosity of water. Since the density of the sedimenting particles was unknown, no correction could be made for the density of the solvent. No correction was made for the viscosity of the solvent, except in two experiments where the viscosity of the whole solution, including the slowly sedimenting substances, was determined separately in an Ostwald viscometer at 25° C.

RESULTS

In early experiments, when the cytoplasmic particles were separated in veronal-chloride buffer at pH 7.8, instead of in sucrose, and resuspended in 5 cc. of buffer per gram of spleen, no boundaries were seen in the sedimentation pattern of normal spleen, and only one very small boundary in the leukemic preparations. When the separations were repeated in 0.88 M sucrose, which has been found to cause less aggregation of submicroscopic particles than do buffer solutions (6), and the particles were resuspended in 2.5 cc. of pH 8.5 buffer per gram of spleen, the sharp boundary was clearly visible in normal spleen preparations and greatly increased in leukemic preparations.

TABLE 1

THE AMOUNTS AND SEDIMENTATION RATES OF THE CYTOPLASMIC COMPONENTS FROM NORMAL AND LEUKEMIC MOUSE SPLEEN

BUFFER			Concentration in mg/gm of fresh tissue						Sedimentation rate in *S**				
Mice	Exp. no.	Hrs. sed.	A	B	C	D	E	B+C+D+E	A	B	C	D	E
Normal Ak	1	2		1.43	←——0.25——→					65			
" "	4a	2		1.76	←——1.16——→			2.92		63			
" "	7	3		2.06	←——0.27——→					67			
Normal CF	2	2		1.92	←——0.80——→					60			
Trans. leuk. Ak	5a	2		3.58	1.44	0.56	1.34	6.92		51	41(80)†		27(53)†
" " "	8	3		5.68	2.08	trace	1.42			63	51(81)		35(56)
" " "	10a	¾‡		1.76	0.31	←0.12→				64			
	10b	3		4.64	1.23	0.59	1.30			57	49(86)		31(54)
Spont. leuk. Ak	6	2		4.72	1.77	1.10	0.64			40	33(83)	28(70)†	22(55)
" " "	9a	3		4.66	1.27	0.87	0.60	7.40		62	50(81)		35(56)
Spont. leuk. C58	3a	2	0.33	2.49	1.27	1.22	1.10		93(158)†	59	44(75)	40(68)	33(56)
Same after 24 hours	3b			2.82	1.10	←0.81→				60	44(73)		
SUCROSE													
Normal Ak	4b	2	1.06	2.03	0.82		0.28	3.13	21.1(139)	15.2	11.5(76)		8.5(56)
Trans. leuk. Ak	5b	2		4.80	1.72		0.54	7.06	15.9§(110)	14.4	11.7(81)		7.9(55)
" " "	11#	3		5.78	2.54		0.59			12.6	10.5(83)		7.6(60)
Spont. leuk. Ak	9b	3		3.15	1.40		trace	4.55	20.6§(145)	14.2	10.8(76)		

* *S* = Svedbergs = cm/sec/unit of force $\times 10^{-13}$.
† Per cent of sedimentation rate of *B*.
‡ At 92,000 × *g*.
§ Band of turbidity.
# Analyzed cold.

The sedimentation patterns were enlarged and traced on graph paper, and the areas under the sedimenting boundaries determined by planimetry or by counting squares. The concentration of each of the components was calculated by the usual procedure (3) from the area under the curve and the optical constants of the apparatus. The refractive index increment was assumed to be 1.87×10^{-4} for a solution containing 1 mg. of protein per cc. The protein concentrations were then corrected to mg/gm of spleen.

Typical sedimentation patterns obtained on particles separated from normal, transplanted leukemic, and spontaneous leukemic spleen in the sucrose medium, and analyzed in buffer, are shown in Figure 1, and the analysis of a leukemic pattern is illustrated in Chart 2. The calculated amounts of the various components are given in Table 1. The particles from normal spleen, (Fig. 1, *N*)

showed one well defined component, *B*, followed by a small amount of heterogeneous material. A similar pattern was obtained on spleen from Carworth Farms mice. In the particles obtained from transplanted leukemic spleen (Fig. 1, *T*), the concentration of component *B* was elevated to at least twice the normal value, and the more slowly moving material was greatly increased in amount and separated into two fairly distinct components, *C* and *E*, with a trace of an intermediate boundary, *D*. In experiment 10, the particles from leukemic spleen were sedimented in two fractions. After centrifuging for 45 minutes at 92,000 × *g*, the pellet contained a significant amount of *B* but only traces of the slower components. The remaining particles, sedimented for 3 hours at 170,000 × *g*, gave the usual leukemic pattern.

The patterns obtained on spontaneous leukemia (Fig. 1, *S*) resemble those found for transplanted leukemia, in that they show a great increase in the concentrations of all the components over the normal values. The amounts of *B* and *C* were similar to those found in transplanted leukemia, while the amount of *D* was greater, and that of *E* considerably less. The single C58 mouse spleen examined showed a similar pattern and, in addition, a small amount of a faster sedimenting component, *A*. This analysis was repeated after the solution had been kept overnight in the cold. The *B* and *C* boundaries were unchanged, but the *A* boundary and over half of *D* and *E* had disappeared.

When the aggregated particles had not been removed by a preliminary centrifugation at 20,000 × *g*, the solution was quite turbid. The material responsible for the turbidity sedimented to the bottom of the cell while the ultracentrifuge was coming up to speed; it appeared to be very polydisperse and showed no definite boundary. When the preparative sedimentation of the particles was prolonged from 2 to 3 hours, no consistent increase in yields resulted (Table 1).

The suggestion has been made (13) that quantities of cellular constituents be expressed as amounts per cell rather than as amounts per unit weight of tissue. No cell counts were made in these experiments. The numbers of cells per gram of normal and leukemic mouse spleen have, however, been determined in this laboratory, by a technic to be reported elsewhere.[2] The average cell counts obtained in that study are given in Table 2, together with the average amounts of components *B*, *C*, *D*, and *E* (from Table 1), and the calculated amounts of these substances per cell. Since both types of leukemic spleen contain fewer cells per gram than the normal tissue, the elevations in the concentrations of the ultracentrifugal components are even more striking on a per cell basis than on a weight basis.

In every analytical experiment a large boundary remained at the meniscus. In one experiment, where the pattern was observed for 70 minutes, the boundary spread considerably, but the steepest gradient still remained at the meniscus. The lighter area at the right of each picture was caused by a slowly sedimenting yellow pigment boundary. In the 14-minute picture of the normal spleen experiment (Fig. 1, *N*4) the position of the pigment boundary coincides with the shoulder on the large boundary. The finding that this material sedimented so slowly in the analytical cell was unexpected, since the fractions had been previously prepared by high-speed centrifugation, and preparations resuspended in sucrose could be sedimented a second time in the preparative rotor. This phenomenon is being investigated further.

In several experiments the particles were centrifuged in two portions. One pellet was resuspended in buffer and the other in 0.88 M sucrose, and both samples were analyzed in the ultracentrifuge. The patterns obtained in sucrose are shown in column 5 of Figure 1, and the analytical results are given in Table 1. The particles from normal spleen showed a small amount of material sedimenting ahead of the main boundary. This has been listed in column *A* of Table 1 for convenience, although it may not be the same as the fast boundary seen in experiment 3*a*. There was a good-sized *B* boundary, a definite *C* boundary, and a small amount of *E*. The patterns from transplanted leukemic spleen showed a band of turbidity moving ahead of the *B* boundary. The *B*

TABLE 2

CALCULATION OF THE AMOUNTS OF CYTOPLASMIC COMPONENTS PER CELL

	No. cells per gram	B		C		D		E	
		Mg/gm	Mg/cell	Mg/gm	Mg/cell	Mg/gm	Mg/cell	Mg/gm	Mg/cell
Normal Ak	23×10^8	1.8	0.8×10^{-9}	←		0.56	0.2×10^{-9}	→	
Trans. leuk. Ak	14×10^8	4.6	3.3×10^{-9}	1.8	1.3×10^{-9}	0.6	0.4×10^{-9}	1.4	1.0×10^{-9}
Spont. leuk. Ak	18×10^8	4.7	2.6×10^{-9}	1.5	0.8×10^{-9}	1.0	0.6×10^{-9}	0.6	0.3×10^{-9}

[2] N. A. Mizen and M. L. Petermann, in preparation.

boundary was spread more than it was in buffer, and increased in size; the *C* boundary was also somewhat larger. No *D* boundary was apparent, and the *E* boundary was much smaller than in buffer. The total amounts of sedimenting material, however (exclusive of *A*), were the same in sucrose as in buffer.

Two attempts were made to analyze the particles from spontaneous leukemic spleen in sucrose. The turbidity band was even more pronounced than in transplanted leukemia (Fig. 1, *S5*), and the sedimenting boundaries were smaller, so that the total amount of material measured was much less than in buffer.

In all these sucrose experiments, where the optical analyses were made at room temperature, the amount of nonsedimentable material and the behavior of the yellow pigment were quite similar to the findings in buffer. One analysis was made on transplanted leukemic spleen in sucrose in the cold. The patterns were similar to those obtained at room temperature, except for an increase in the size of the *C* boundary (Exp. 11, Table 1) and the behavior of the yellow pigment, which now sedimented with the *B* boundary instead of remaining near the top of the cell.

The sedimentation rates found for the various components in buffer are given in Table 1. They proved to be extremely variable, the values for *B* ranging from 40 to 67 *S*. Whatever their sedimentation rates, however, components *C*, *D*, and *E* always traveled at the same rates relative to *B; C* moved about 80 per cent as fast as *B*, *D* about 70 per cent as fast, and *E* about 55 per cent as fast. It therefore seems likely that the wide variations in sedimentation rate were caused by variations in the viscosity of the solution, since the sedimenting particles were moving through a medium which contained all the slowly sedimenting material as well as some sucrose. In two experiments, 7 and 8, the relative viscosity of the solution was measured at 25° C. on particles sedimented for 3 hours, resuspended in buffer, and cleared of aggregated material at 20,000 × *g* in the usual way. The relative viscosities were only 1.13 for normal particles and 1.14 for leukemic particles, and the sedimentation rates of *B* (measured at the same time of day) were relatively high, 67 and 63 *S*. Corrected for the viscosity, the sedimentation constants were 76 for the normal particles and 72 for the leukemic. Until the viscosity has been determined on samples of low sedimentation rate (such as experiment 6), the effect of viscosity on the sedimentation rate cannot be definitely determined.

The sedimentation constants in sucrose were much lower than those obtained in buffer. No attempt was made to correct them for the viscosity of the sucrose, since no density correction could be made. The relative sedimentation rates of *B*, *C*, and *E* were the same as in the buffer.

In one experiment normal spleen was homogenized and centrifuged in the phosphate-heparin buffer suggested by Hoster and associates (7). No trace of any of the rapidly sedimenting boundaries was seen in the ultracentrifuge pictures.

DISCUSSION

The question of how well the analytical results reported here represent the actual amounts of these substances originally present in the spleen is difficult to answer. Although the homogenization procedure used in this laboratory (3 minutes per gram of spleen, in a Potter-Elvehjem homogenizer [10]) was worked out under microscopic control, to leave the nuclei intact while breaking as many cell membranes as possible, many whole cells remained and were discarded with the nuclear fraction. While the yield of cytoplasmic constituents cannot therefore be considered quantitative, extensive experience in this laboratory has shown that, with a consistent homogenization procedure, the nitrogen distribution between the nuclear and cytoplasmic fractions is quite similar for normal and leukemic spleen (10), showing that about the same percentage of cells has been broken in both cases. Some error has also been introduced in the calculation of mg of protein/gm of spleen. When one pellet was resuspended in 2.5 cc. of buffer, the total volume of the sample was assumed to be 3.0 cc. Variations in the amount of fluid left on top of the pellet may have introduced errors here of the order of 10 per cent.

The lack of stability of the observed components must also be considered. When the spleens were homogenized and fractionated in veronal-saline buffer at pH 7.8, there was no trace of a sedimenting boundary in the normal spleen preparations, and only a very small boundary in the leukemic particles; likewise, the one normal preparation made in phosphate-heparin buffer at pH 8.0 showed no boundary at all. The nucleoprotein of sedimentation constant 79 *S* isolated from chick embryo bodies by Taylor and associates (16) was most stable in distilled water at pH 7.0; 0.005 M electrolyte and higher alkalinity both favored the break-up of the *S* 79 component into more slowly sedimenting substances. That the slower components observed here were not merely breakdown products of the largest component is suggested by several lines of evidence. First, in one

experiment where the particles were separated into a fraction sedimenting in 45 minutes and one sedimenting in 3 hours, the first fraction was relatively free of the slower components, which were concentrated in the second fraction. Second, when a particle suspension was analyzed after standing overnight in buffer (Exp. 3*b*, Table 1), the concentrations of the faster components, *B* and *C*, were about the same, while *D* and *E* had decreased to about one-half the concentrations found on the first day. Finally, components analogous to *C* and *E* have recently been observed in fresh extracts of chick embryo tissue (19).

Because of the instability of these preparations, all the experiments reported here have been completed within 10–12 hours of the death of the animals. Even within this period, changes must have taken place in some of the particles, since a small amount of the material (which had originally sedimented at 170,000 × *g*) could be sedimented at 20,000 × *g*. Although the solutions were always kept cold until just before filling the cell, the possibility remained that, in the half hour that elapsed between the warming of the sample and the first picture, the particles had changed their physical state. One optical run was therefore carried out in the cold. It showed the same amounts of sedimenting and nonsedimenting material as were found in the analyses made at room temperature; the principal difference observed was in the behavior of the pigment, which appeared to be bound to component *B* in the cold but dissociated after only 30 minutes at room temperature.

The size of these particles cannot be calculated without knowing their density and shape. For the chick embryo body component with a sedimentation constant of 79 *S* and a density of 1.27 the diameter was calculated to be 23 mμ (16). This agreed fairly well with the value of 18 mμ obtained from electron micrographs (17). The diameter of the spleen particles of sedimentation constant 72–76 *S* is probably of the same order of magnitude, about 20 mμ, if they are spherical.

The significance of these macromolecular components in the cytoplasm and of their increased concentration in leukemia is unknown. The small amounts of these substances found in the spleens of "normal" Ak mice cannot be considered characteristic of a preleukemic state, since a similar pattern was obtained on Carworth Farms mice, in which leukemia does not occur. Particular interest is attached to the fact that these substances are elevated in spontaneous leukemia. In earlier studies made in this laboratory, the elevations in pentosenucleic acid content found in the cytoplasm (10) and nuclei (11) of transplanted leukemic spleen were not found in spontaneous leukemia,[3] so that doubt arose as to whether the changes observed were attributes of the neoplastic state or merely characteristic of rapid cell growth. In the experiments reported in this paper, the changes found in spontaneous leukemia are just as great as those observed in transplanted leukemia.

Particles prepared from normal and transplanted leukemic spleen have also been compared by immunological methods (4). It is interesting to speculate that the antigen responsible for the increased cytotoxic activity of the antiserum to the leukemic particles may have been one of the components described here.

Particles in the same size range as component *B* (with sedimentation rates of 60–80 *S*) have been found in rat liver (15), monkey spinal cord (9), rabbit and human embryo brain (17), and chick allantoic fluid (8), embryo body (16), and embryo brain (17). Similar particles were also found (in much smaller amounts than in embryonic brain) in adult rabbit, human, and chick brain (17). They therefore seem to be widely distributed in animal tissues.

In addition to these sedimentation studies, there have also been a number of reports of cytoplasmic particles observed by electron microscopy. While an increased concentration of particles of 20-mμ diameter has been found in Hodgkin's disease lymph nodes (7), the particles observed in various types of tumors (1, 5, 12, 14) are much larger, ranging from 40 to 250 mμ in diameter.

The leukemic lymphocyte resembles the primitive normal white cell in its morphology and also in its increased pentosenucleic acid content (18). The increased amounts of a substance resembling component *B* found in embryonic as compared to normal brain suggest the possibility that the elevation in these components described here for leukemic spleen may be a further point of resemblance between the leukemic lymphocyte and the primitive normal white cell.

SUMMARY

The macromolecular nucleoprotein particles from the cytoplasm of normal and leukemic mouse spleen have been separated by differential centrifugation and analyzed in the ultracentrifuge. Several well defined sedimentation boundaries have been observed. They represent components whose concentration, either on a weight or on a per cell basis, is greatly increased in both transplanted and spontaneous leukemia.

[3] M. L. Petermann and A. M. Larack, unpublished.

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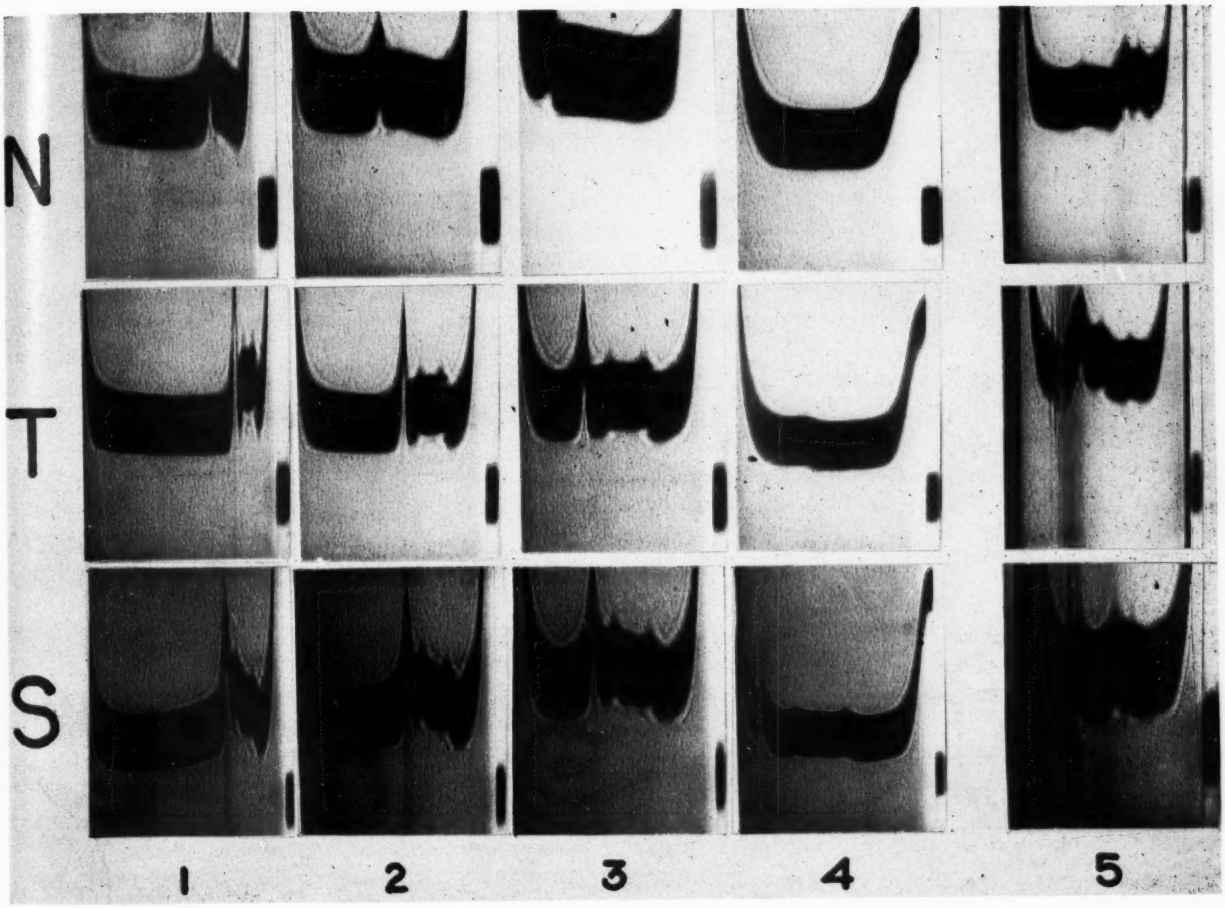
Fig. 1.—Sedimentation patterns of normal and leukemic spleen particles. The direction of sedimentation is to the left.

N.—Normal Ak spleen: 1 to 4, in buffer, after 0,4,8, and 28 minutes at 52,640 r.p.m. (Exp. 4a); 5, in sucrose, after 24 minutes at 59,780 r.p.m. (Exp. 4b).

T.—Transplanted leukemia. 1 to 4, in buffer, after 1,5,9, and 19 minutes at 52,640 r.p.m. (Exp. 5a); 5, in sucrose, after 28 minutes at 59,780 r.p.m. (Exp. 5b).

S.—Spontaneous leukemia, Ak. 1 to 4, in buffer, after 3, 7, 11, and 17 minutes at 52,640 r.p.m. (Exp. 6); 5, in sucrose, after 23 minutes at 59,780 r.p.m. (Exp. 9b).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Regression or Survival of Tumor Homoiografts in Mice Pretreated with Injections of Lyophilized Tissues*†

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It has been shown that tumor homoiografts between mice of unrelated inbred strains (which usually regress) will grow successfully if the hosts are injected with lyophilized mouse tissues or their antisera prior to inoculation of the grafts (3, 5, 10, 11). It has also been reported that, under certain conditions, inhibition of the grafts followed such treatment (4, 10, 11).

Two alternative hypotheses may be advanced to explain the mode of action of the prior injections leading to the opposite effects. The first is based on the assumption that we are dealing with two distinct classes of substances, either growth-inhibiting or growth-stimulating in nature, which act directly on the graft.

The second hypothesis would be the assumption that the effect of the prior injections is upon the host, altering its responses to the "foreign" graft. This change in reaction may be expressed on the one hand by successful resistance of the host to the graft ("inhibition" of the graft) and on the other by the break-down of resistance of the host (apparent "stimulated" growth of the graft).

The experiments to be reported here were designed to attempt to differentiate between these two hypotheses and to arrive at some understanding of the nature of the underlying phenomena leading to the observed "inhibition" or "enhancement" of tumor homoiografts. It was postulated that, with a suitable selection of materials, prior injection of the lyophilized tissues in widely differing dosages might offer a clue to the mechanisms underlying the observed differences.

MATERIALS AND METHODS

Three transplantable mouse tumors were used as the test grafts. The host strains of mice chosen were those in which it was known, from previous experience, that the test tumors normally grew in approximately 50 per cent of the untreated animals. Such a choice would afford a base-line to detect the alternative possibilities either of an increased number of "takes" or of regression of the grafts in the experimental (treated) animals. All the animals were about 2–3 months old at the start of the experiments and were about equally divided by sex. All females were virgins. The mice were housed with a maximum of five or six to a cage, and were fed Purina Fox Chow and water ad libitum. No histories concerning infection were kept during the experiment, but all the animals appeared healthy and vigorous at the start.

The materials to be injected were lyophilized normal or cancerous mouse tissues. These were removed under aseptic conditions and kept at the temperature of melting ice until they were frozen for lyophilization. Freezing was done rapidly over dry ice, and the tissues were then lyophilized. The maximum interval between securing the tissues and the initiation of freeze-drying was about 1 hour. After drying, the tissues were powdered and sealed under vacuum in ampoules. The sealed ampoules were stored under refrigeration (about 5° C.).

For purposes of injection, the lyophilized materials were resuspended and homogenized in sterile 0.85 per cent saline or sterile double-distilled water. The suspensions were kept cold for injection. All injections were made intraperitoneally in 0.5-ml. amounts. Injections were given in equal amounts twice weekly for a total of five injections. One week after the last injection, a single inoculation of bits of minced live tumor was made subcutaneously by trocar, in the right suprascapular region, under sterile conditions.

The course of growth of the grafts was followed by palpation, either until the animals died with tumors or were recorded as negative for a consecutive period of at least 2 months. Animals bearing no tumors were then discarded.

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Description of Transplantable Tumors and Inbred Lines of Mice

(The description of the tumors is based on information kindly supplied to us by Dr. Elizabeth Fekete of this laboratory.)

Tumor E 0771 is a mammary gland adenocarcinoma which arose spontaneously in a C57 black/6 female at the Jackson Laboratory in 1939. It is a virulent tumor, killing 100 per cent of C57 black/6 hosts (both males and females) within 2–3 weeks after implantation. The host test strain was the C3H/Ks line which is a subline of the C3H strain.

TABLE 1

THE EFFECT OF PRIOR INJECTIONS OF LYPHOLIZED TUMOR E 0771 ON THE SUBSEQUENT GROWTH OF GRAFTS OF TUMOR E 0771 IN C3H/Ks MICE

Group no.	Total injected per mouse (mg. dry wt.)	Mice dying with tumors No.*	Per cent
Experiment 1:			
1	50	15/17†	87
2	5	13/19	68
3	0.5	7/13	54
4	0.05	4/20‡	20
5	Control	8/17	47
Experiment 2:			
6	50	17/18†	94
7	5	18/18†	100
8	0.5	8/18	44
9	0.05	9/20	45
10	0.005	5/20‡	25
11	Control	10/18	56
Experiment 1 plus 2:			
4, 9, 10	0.05 and 0.005	18/60§	30
5, 11	Control	18/35	51

* Numbers in the numerator are the number dying with tumors; numbers in the denominator are the total number of mice in each group.

† The probability (P) is 1 per cent throughout, or less, that this result could have been obtained by chance.

‡ P is just over 5 per cent.

§ P is less than 5 per cent.

Tumor C 1300 is a round-cell tumor, possibly a neuroblastoma. It arose spontaneously in a strain A mouse in 1940 at the Jackson Laboratory. It grows in 100 per cent of strain A males and females, death occurring within 2–3 weeks after implantation. The test strain used as host was the BALB/cJax, which traces back to the Bagg albino line.

Tumor 15091a is an anaplastic mammary carcinoma, predominantly spindle-celled, which arose spontaneously in a strain A female at the University of Michigan in 1928. It grows rapidly in 100 per cent of strain A males and females, death occurring within 3–5 weeks after implantation. The test strain used as host was the ST/Ks. This is a subline of the Street strain.

RESULTS

Experiments with the C57 black/6 tumor E 0771.—Table 1 presents the data for the effect of prior injections of lyophilized tumor E 0771 on the growth of grafts of tumor E 0771 in C3H/Ks mice. Two experiments are reported. The lyophilized tissue used for Experiment 1 was prepared from a different batch of tumors from that used for Experiment 2. The total amounts of lyophilized tissue injected per mouse range from 50 mg. down to 0.005 mg. dry weight.

It is clear that the mice receiving the larger doses (5 mg. and 50 mg.) showed a significant increase in the number of tumor "takes," as compared to the control (uninjected) animals, while the smaller doses (0.05 and 0.005 mg.) appear to have led to inhibition of tumor growth. This is more clearly brought out when the combined data for the latter groups in Experiments 1 and 2 are compared to those for the combined controls.

Table 2 presents the results of varying the amounts injected of two lyophilized normal tissues, kidney and liver, from C57 black/6Ks mice. The largest dose of kidney (50 mg.) led to enhanced growth of the grafts, while the smallest dose (0.005 mg.) led to inhibition. The injections of lyophilized liver had no effect, either of enhancement or inhibition.

TABLE 2

THE EFFECT OF PRIOR INJECTIONS OF LYOPHILIZED KIDNEY AND LIVER FROM C57 BLACK/6Ks MICE ON THE SUBSEQUENT GROWTH OF TUMOR E 0771 IN C3H/Ks MICE

Lyophilized substance injected	Total injected per mouse (mg. dry wt.)	Mice dying with tumors No.*	Per cent
B/6Ks kidney	50	9/10†	90
	5	4/10	40
	0.5	3/10	30
	0.05	3/10	30
	0.005	0/9‡	0
B/6Ks liver	50	5/10	50
	5	5/10	50
	0.5	5/10	50
	0.05	5/10	50
	0.005	3/9	33
Control (0.85 per cent saline)		4/10	40

* Numbers in numerator are the number dying with tumors; numbers in the denominator are the total number of mice in each group.

† The probability (P) is less than 2 in 100 that this value differs from the control group by chance.

‡ P is less than 5 per cent.

Experiments with the strain A tumor 15091a.—Table 3 presents the data for the effects of prior injections of lyophilized tumor 15091a on the growth of grafts of this tumor in ST/Ks (Street strain) mice. Two experiments are reported. The lyophilized tissue used for Experiment 1 was prepared from a different batch of tumors from that used for Experiment 2.

In Experiment 1, the smaller number of mice in Groups 3, 4, and 5 is due to accidental loss not

connected with the experimental procedure. It is clear, particularly from Experiment 2, that the larger doses (50 and 5 mg.) of lyophilized tumor led to enhanced tumor growth, while the smaller doses (0.05, 0.005, and 0.0005 mg.) led to inhibition of tumor growth. Certain of the experimental groups (1, 4, 8, 9) did not differ significantly from the controls. Groups 2 and 3 of Experiment 1 are just above the 5 per cent level in the probability that the observed results could have been obtained by chance.

Experiments with tumor C 1300.—Table 4 presents the data for the effects of prior injections of lyophilized tumor C 1300 on the growth of grafts of this tumor in BALB/cJax mice. The largest dose (5 mg.) led to a significant increase in the number of takes, while the smaller doses (0.05 and 0.005 mg.) led to a significant decrease in the number of takes. There was no significant difference between the control group and the groups receiving injections of either 0.5 mg. or 0.0005 mg. of lyophilized tumor.

TABLE 3

THE EFFECT OF PRIOR INJECTIONS OF LYOPHILIZED TUMOR 15091A ON THE SUBSEQUENT GROWTH OF GRAFTS OF TUMOR 15091A IN ST/KS MICE

Group no.	Total injected per mouse (mg. dry wt.)	Mice dying with tumors No.*	Per cent
Experiment 1:			
1	50	12/18	66
2	0.5	2/15†	13
3	0.005	1/9†	11
4	0.0005	1/6	17
5	Control (saline)	4/8	50
3, 4		2/15†	13
Experiment 2:			
6	50	20/20‡	100
7	5	15/21‡	71
8	0.5	9/22	41
9	0.05	3/22	14
10	0.005	1/21§	5
11	0.0005	0/20‡	0
12	Control (nothing)	6/21	29
10, 11		1/41‡	2

* Numbers in the numerator are the number dying with tumors; numbers in the denominator are the total number of mice in each group.

† The probability (P) is just over 5 per cent that this value could have been obtained by chance.

‡ P is less than 1 per cent.

§ P is between 2 per cent and 5 per cent.

DISCUSSION

For the conditions of the experiments reported above, it is clear that the larger doses of lyophilized tissues prior to inoculation of the tumor homoiografts cause a significant increase in the number of "takes," while the small doses lead to inhibition of growth of the grafts. The groups receiving amounts intermediate between the large and small doses do not differ from the controls in the number of takes. The magnitude of the diverse effects varies from tumor to tumor, being most marked for tumor C 1300 and least marked for tumor E 0771.

These observations support the hypothesis that we are dealing with a single type of substance (or category of substances) in the lyophilized tissues which, depending upon the amount of tissue injected, serve to alter the reactions of the host in the direction of either decreased or increased resistance to the "foreign" tumor graft. Such an interpretation is further strengthened by the fact that the groups of mice receiving the "intermediate" doses did not differ significantly from the controls in the number of takes. It is of interest in this connection that when normal tissues were used (Table 2) the lyophilized liver produced no effect at all, while the results with the kidney paralleled those with the tumor tissues. Also, as might be expected a priori, a limit is reached in the minimum amount of material that may be injected beyond which there is again no effect upon the host. This is shown in Table 4, where the group receiving 0.0005 mg. of lyophilized tumor did not differ significantly from the controls.

TABLE 4

THE EFFECT OF PRIOR INJECTIONS OF LYOPHILIZED TUMOR C 1300 ON THE SUBSEQUENT GROWTH OF GRAFTS OF TUMOR C 1300 IN BALB/CJAX MICE

Total injected per mouse (mg. dry wt.)	Mice dying with tumors No.*	Per cent
5	26/30†	87
0.5	17/29	59
0.05	4/30†	13
0.005	5/30†	17
0.0005	12/30	40
Control	15/30	50

* Numbers in the numerator are the number dying with tumors; numbers in the denominator are the total number of mice in each group.

† The difference between this value and the control group is statistically significant. The probability is less than 1 in 100 that it could have been obtained by chance.

Whether there will be any effect of the injections at all appears to depend in the first instance upon the specific host-graft relationships, as expressed in the untreated animals. This point is demonstrated by data from several of our unpublished experiments. Thus, a significant increase in the number of takes in animals receiving the larger doses of lyophilized homologous tumor and no difference between controls and animals receiving the small doses were found when Sarcoma 180 was tried in ST/Ks mice. Similar results were obtained when tumor 15091a was tried in DBA/1Jax mice and in a most recent test of tumor E 0771 in

C3H/Ks mice. In the latter two instances, the relations between host and graft had changed in time, so that only 28 per cent of the DBA/1Jax mice and 20 per cent of the C3H/Ks mice had takes, whereas approximately 50 per cent of untreated animals had died with tumors in previous tests.

Variability in results may also derive from the empirical nature of our experimental procedure, wherein the dry weight of a complex tissue of unknown chemical constitution is used as the basis of dosage. This may be expressed in variations between lyophilized lots of the same type of tissue prepared at different times. Such a possibility is suggested by the data of Tables 1 and 3. In Table 1, the two groups in Experiments 1 and 2 receiving a total dosage of 5 mg. each, differ significantly from each other in the numbers dying with tumors. The same is true for the groups shown in Table 3, which received a total dosage of 50 mg. each.

Our interpretation, that we are dealing with a single type of substance (or category of substances) acting with different effects upon the host, is supported by reports in the literature of similar observations. For the Brown-Pearce rabbit tumor (6), repeated prior intratesticular injections of Berkefeld filtrates of the tumor resulted in a greater susceptibility of the rabbits to subsequent grafts of the live tumor, while prior subcutaneous injections of the filtrates in higher dilutions resulted in increased resistance to the tumor and greater logevity of the hosts. Casey (1) reports an observation of inhibition of the Brown-Pearce tumor in rabbits followed a single intratesticular injection of Berkefeld filtrate. This is in contrast to the usual result of enhanced growth of the tumor following similar treatment (2).

A parallel is suggested between our results and the reported effects of injections of tissue antisera on the healing of experimentally produced wounds in mice and guinea pigs (7–9) and fractures in rabbits (12). The small doses of antisera accelerated healing, while the larger doses resulted in a depression of healing rate, as compared to the controls. The antisera were injected after the wounds or fractures were made, in contrast to our procedure of injections prior to grafting. (We have already reported [3] the enhancing effect of prior injections of tissue antiserums on tumor homoiografts in mice.)

In conclusion, it must be emphasized that our experiments were conducted with transplantable tumors, and thus involved host-graft reactions which may not necessarily derive from the fact that we were dealing with tumors per se. Whether similar effects would be obtained with spontaneous tumors or with normal tissue grafts is yet to be determined.

SUMMARY

1. Prior injections of homologous lyophilized mouse tumors, or a normal tissue, into the hosts lead to a significant increase or decrease in the number of "takes" of tumor homoiografts. Enhanced tumor growth follows the large doses, while inhibition follows the smaller doses. The intermediate doses do not have any effect.

2. It is postulated that we are dealing with one substance (or category of substances) in the lyophilized tissue, whose injection in different dosages serves to change the "normal" responses of the host to the graft, leading to either an increase or a decrease in resistance to the graft.

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Influence of Sarcoma 180 on Adenocarcinoma 755 in the Mouse*

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The growth rates of tumors, although influenced in part by local vascular conditions, appear to depend on the genetic endowments and the metabolic states of both the tumor and the host. There is abundant evidence which indicates that the host-tumor relationship embraces more than local phenomena, and that, in addition to competition for metabolites, both tumor and host exert reciprocal influences on each other. The generalized effects of tumors of endocrine glands which elaborate hormonal substances, particularly those of pituitary, adrenal, and pancreatic islets, are well known. The possibility that tumors of an apparently nonendocrine nature may also produce systemic effects remains to be explored. It is not improbable that some of the determinate properties of malignancy are to be found in the systemic effects of tumors as well as in their local cellular pattern. As a corollary to this, various tumors will affect a host in different ways, possibly antithetical, and two different tumors in the same host might well affect each other.

This paper reports observations on the growth of both Carcinoma 755 and Sarcoma 180 in the same host. Sarcoma 180 is insensitive as to host strain, growing readily in all stocks of mice. Adenocarcinoma 755 is strain-fixed to C57 black mice. The differences between these two tumor types are demonstrated also by their individual responses to 8-azaguanine, which is markedly inhibitory to Carcinoma 755, but wholly without effect on Sarcoma 180 (2).

EXPERIMENTAL

Approximately 300 mice, 4–5 months of age, both male and female, of the C57 strain inbred in these laboratories for many generations, were used in these experiments. Animals in a single experiment received simultaneous (by trocar) implants of approximately equivalent fragments from a single freshly dissected tumor free of necrosis. Carcinoma 755 was implanted in the right axilla, and, 12–15 days later, Sarcoma 180 was implanted in the left axilla. Additional control animals received implants of only one of the tumors. The animals were killed 7–10 days after the last implantation and the freshly dissected tumors weighed individually. The timing of operations is especially important because of the rapid rate of growth of Sarcoma 180 as compared to that of Carcinoma 755. In order to observe the activity of the drug in a situation where both a susceptible and a resistant tumor were present, half the animals in one experiment were treated with 8-azaguanine, 2 mg/day, from the time of the second implantation to the conclusion of the experiment.

The tumor weights from the several experiments cannot be compared to one another, since the experiments were carried out at different times and with different donor tumors. The sex differences from one experiment to another have no significance, it having been observed previously that each of the tumors grew equally in both sexes after implantation from the same batch of donor tumor.

DISCUSSION

The tumor weights in the several experiments (Table 1) consistently show that Sarcoma 180 inhibits the growth of Carcinoma 755. Conversely, the growth of Sarcoma 180 appears to be enhanced by the co-existence of Carcinoma 755. This apparent stimulatory effect of the carcinoma on the sarcoma is not abolished by treatment with 8-azaguanine, indicating that it does not involve the mechanism susceptible to interference by azaguanine. On the other hand, the inhibitory effects of Sarcoma 180 and of 8-azaguanine on Carcinoma 755 are additive. 8-Azaguanine did not affect Sarcoma 180 even in the mice in which Carcinoma 755 was also present.

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That the growth rate of Carcinoma 755 is not limited by the capacity of the host reservoir to supply essential nutrients had been previously demonstrated by the finding that the same host can support at least three implants of Carcinoma 755 at different sites, the individual tumors growing at the same rate as corresponding singly implanted controls.

The data suggest the possibility that Sarcoma 180 elaborates a substance to which Carcinoma 755 is sensitive; conversely, Carcinoma 755 might elaborate a substance beneficial to Sarcoma 180 or alter the host so as to favor the growth of Sarcoma 180. That the proliferative influences which are accelerated in regenerating liver are blood-borne has been demonstrated by Bucher, Scott, and Aub (1). Whether or not the sarcoma-stimulatory effect of Carcinoma 755 is of a similar nature is yet to be explored. Whatever the course of events, these findings provide further evidence of the fundamental biological differences between the two tumors and illustrate again the humoral or systemic factors in neoplastic processes.

The histology of the visceral organs of the experimental animals is under study and will be reported elsewhere.

SUMMARY

Two different experimental tumors, adenocarcinoma 755 and Sarcoma 180, have been grown in the same host and some of their reciprocal effects observed.

Sarcoma 180 appears to inhibit the growth of Carcinoma 755, but Carcinoma 755 enhances the growth rate of Sarcoma 180.

TABLE 1

INFLUENCE OF SARCOMA 180 ON CARCINOMA 755

No.	Tumor	Sex	No. of mice	Weight of Sarcoma 180 (gm.)	Weight of Carcinoma 755 (gm.)
I	180	F	20	0.992±0.245	
	755	F	20		1.509±0.539
	180/755	F	20	1.559±0.280	0.686±0.307
II	180	M	20	1.060±0.210	
	755	M	19		0.550±0.424
	180/755	M	20	1.762±0.217	0.314±0.176
III	180	F	17	0.985±0.185	
	755	F	17		1.416±0.325
	180/755	F	17	1.468±0.246	0.432±0.270
IV	180	M	20	0.876±0.205	
	755	M	20		0.763±0.390
	180/755	M	20	1.398±0.215	0.400±0.220
V	180	M	9	0.800±0.225	
	755	M	10		1.658±0.118
	180*	M	10	0.816±0.182	
	755*	M	10		1.030±0.160
	180/755	M	8	1.400±0.325	0.951±0.160
	180/755*	M	18	1.266±0.239	0.327±0.165

* Treated with 8-azaguanine.

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Announcements

Gordon Research Conferences, AAAS

Cancer Conference

The Gordon Research Conferences, sponsored by the AAAS, for 1952, will be held from June 16 to August 29 at Colby Junior College, New London, New Hampshire. The facilities of the school have been made available for the conferences.

The Gordon Research Conferences, AAAS, were established to stimulate research in universities, research foundations, and industrial laboratories. This purpose is achieved by an informal type of meeting consisting of the scheduled lectures and free discussion groups. Sufficient time is available to stimulate informal discussions among the members of a Conference. Meetings are held in the morning and in the evening, Monday through Friday, with the exception of Friday evening. The afternoons are available for recreation, reading, resting, or participation in discussion groups as the individual desires. This type of meeting is a valuable means of disseminating information and ideas which otherwise would not be realized through the normal channels of publication and scientific meetings. In addition, scientists in related fields become acquainted, and valuable associations are formed which result in collaboration and co-operative effort between different laboratories.

The first meeting of each Conference is held Monday morning at 9 o'clock, Eastern Daylight Saving Time. The morning sessions, through Friday, are scheduled from 9:00 A.M. to 12:00 noon. The second session of each day is held in the evening from 7:30 to 10:00 P.M., Monday through Thursday. There are no Friday evening meetings. Conference members are expected to release their rooms not later than Sunday morning unless they have made a reservation for the next Conference.

Accommodations are available for a limited number of women to attend each conference, and also for wives who wish to accompany their husbands. All such requests should be made at the time of the request for attendance, because these limited accommodations will be assigned in the order that specific requests are received. Children 12 years of age and older can be accommodated at Colby Junior College. Rooms are available at several inns and hotels in the area if reservations are made in advance. No dogs or other animals will be permitted in the School dormitories.

Individuals interested in attending the Conference are requested to send their applications to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island, on or before May 15, 1952. From June 15 to September 1, 1952, mail should be addressed to Colby Junior College, New London, New Hampshire. Attendance at each Conference is limited to 100.

The program for the 1952 Cancer Conference will be:

August 25:

Georgiana Bonser. A study of the Aromatic Amines in Relation to Carcinogenesis with Special Reference to Betanaphthylamine.

C. Lenore Simpson. Trypan Blue-Induced Tumors of Rat Liver.

Arthur Vorwald. Pulmonary Cancer Induced by Beryllium Compounds Deposited in the Lungs of White Rats.

Joseph Beard. Avian Leucosis: A Template for Cancer Research.

August 26:

George Palade. Recent Progress in the Study of Cell Structure.

Cyrus Barnum. Some Aspects of the Chemical and Metabolic Heterogeneity of Cytoplasm.

Alexander Dounce. Chemistry of the Nucleus and Nucleolus.

J. Murray Luck. The Incorporation of Labeled Amino Acids by Liver Histone—*in vitro* and *in vivo* Studies.

Henry Hoberman. Studies on the Kinetics of Formation of Nuclear Proteins.

August 27:

Charles Heidelberger. Studies on Nucleic Acid and Protein Biosynthesis in Tumor-bearing Rats.

Leon Miller. Studies in Protein Synthesis in Experimental Tumors.

Henry Lemon. Comparison of Proteins in Human Neoplasms and Homologous Tissues of Origin. A. Acid Phosphomonoesterases, B. Desoxypentose Nucleoproteins.

The evening session will be a round-table discussion.

August 28:

Arthur Walpole. Association among Growth Inhibitory, Carcinogenic and Mutagenic Activity in Ethyleneimine Derivatives.

Erich Hirschberg. Studies on the Mechanism of Action of 8-Azaguanine, Cortisone, and Nitrogen Mustard.

Joseph Leiter. The Action of Compounds Derived from Podophyllin on Tissue Function and Metabolism.

Alice Moore. Subject to be announced.

Joseph Ross. The Mechanism of Anemia Associated with Neoplastic Disease.

August 29:

Leon Jacobson. Evidence for a Humoral Factor (or Factors) Concerned in Recovery from Radiation Injury.

Arthur Kirschbaum. Host Factors in Resisting the Development of Radiation-Induced Neoplasms and Radio-Resistance of Experimental Lymphomas.

G. Burroughs Mider, *Chairman*
Alfred Gellhorn, *Vice-Chairman*